THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

PEYTON ROUS, M.D. HERBERT S. GASSER, M.D.

VOLUME 66, No. 1 JULY 1, 1937

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INFECTIOUS MYXOMATOSIS OF RABBITS

Preparation of Elementary Bodies and Studies of Serologically Active Materials Associated with the Disease

By THOMAS M. RIVERS, M.D., and S. M. WARD

(From the Hospital of The Rockefeller Institute for Medical Research)

Plates 1 and 2

In order to secure further knowledge of the nature of different viruses and to investigate the immunological relation of closely related ones, it is advantageous if not absolutely essential that the infectious agents be obtained in a relatively pure state in amounts adequate for thorough study. The manner in which this can be accomplished varies with the virus under investigation. Stanley (1) has demonstrated that precipitation and crystallization are methods whereby large amounts of certain plant viruses can be obtained. Ledingham (2) showed that by differential centrifugation the elementary bodies of vaccinia and fowl pox, which either represent the infectious units of these maladies or with which they are intimately associated, can be obtained in a state sufficiei   re for purposes of serological investigations in which a coversli̦ technique is used. Craigie (3) also used differential centrifugation ;or the collection of elementary bodies of vaccinia, but his method is more practicable than Ledingham's in that large amounts of the bodies in a relatively pure state can be obtained with ease and regularity. Parker and Rivers (4) have made extensive use of Craigie's method in their chemical and immunological studies of the elementary bodies of vaccinia.

The discovery by Berry and Dedrick (5) that the Shope fibroma virus can be transformed into the agent causing infectious myxomatosis of rabbits, and the fact that Hurst (6) by intracerebral passages

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of the myxoma virus gradually changed it into a neuromyxoma virus different in certain respects from the other two, have focussed attention on these active agents and made it desirable that one or all of them be obtained in amounts and in a state of purity suitable for extensive chemical and serological studies. Indeed, Ledingham (7) has already reported that he is able to secure elementary bodies from tissues infected either with the myxoma virus or with the fibroma virus, in amounts sufficient for slide agglutinations the results of which indicate a serological relation between the bodies from the two diseases.

A number of years ago, Rivers (8) showed that myxoma virus not only attacks subcutaneous tissues but also infects epithelial cells of the skin; this involvement of the epithelial cells manifests itself by the appearance of cytoplasmic inclusions, hyperplasia, and necrosis. In view of these facts it seemed logical to use extensive areas of properly prepared epithelial cells in attempts to obtain large amounts of elementary bodies of myxoma by a technique similar to that devised by Craigie (3) and later used by Parker and Rivers (4) for the collection of elementary bodies of vaccinia. The purpose of this paper is to describe the method used for the collection and purification of elementary bodies of myxoma and to record the results of some serological reactions obtained with them and with materials freed from them.

Materials and Methods

Rabbits.—Large rabbits without defects in the skin were employed. Since work with vaccine virus is also being conducted in the laboratory, only rabbits that had recovered from a vaccinal infection were used for the preparation of elementary bodies of myxoma. This precaution was found by experience to be essential because the myxoma virus became contaminated with vaccine virus when the precaution was not observed.

Virus.—The myxoma virus used in this work was obtained in 1926 from Dr. A. Moses of Brazil through Dr. C. E. Simon; it is the strain with which Shope, Berry, and Hurst have conducted their experiments.

Titration for Infectivity of Virus.—The titer of the infectivity of the virus was determined by intradermal inoculations in rabbits of 0.25 cc. of serial tenfold dilutions of the materials being tested.

Centrifugation.—All angle centrifugation was conducted in a cold room at a temperature of about $+1°C$.

Preparation of Elementary Bodies

Elementary bodies of infectious myxomatosis of rabbits can be prepared in the following manner.

After the fur over a large part of the back and flanks has been removed by clippers, the skin is soaped and carefully shaved; the soap is removed by thorough washing. Then a 10 per cent, bacteria-free emulsion of subcutaneous nodules produced by myxoma virus is applied to the shaved surface with a small pad of 100 mesh wire held by means of a hemostat. Shaving and scarification must not be severe, as too great injury to the skin results in excessive formation of scabs. During the 1st day after scarification the skin is diffusely red. The redness disappears, and on the 3rd day thickening of the skin, at first often in discrete areas, is observed. Then the skin gradually becomes more generally thickened and erythematous until by the 5th day the entire shaved surface is very thick, dark red in color, and marked by folds. Through experience it has been found that the 5th day after inoculation is the best time for harvesting the elementary bodies.

The animal is killed by intravenous injection of air and the infected skin is removed in one piece, care being taken not to get the outer surface soiled with blood; it is then thoroughly stretched and pinned down on a board. Frequently the surface is covered with thin dry crusts which one can easily remove by moistening the surface with ether and brushing it lightly with a towel. 10 cc. of a 0.004 M citric acid-disodium phosphate buffer solution, pH 7.0–7.2, is poured over the surface which is then scraped fairly vigorously with a scalpel. The yellowish cloudy material is collected by means of a spoon, and the procedure is repeated with another 10 cc. of the buffer solution. The combined scrapings are shaken vigorously and spun for 5 minutes at 3000 R.P.M. in a horizontal centrifuge. The supernatant fluid is saved and the sediment is thoroughly shaken with 5 cc. of buffer solution. This suspension is spun 5 minutes at 3000 R.P.M. in the horizontal centrifuge; the supernatant fluid is added to the first and the sediment is discarded. The pooled supernatant fluids are again spun 5 minutes at 3000 R.P.M. in the horizontal centrifuge. The supernatant fluid, from which most of the large particles of debris have thus been removed, is then transferred to flat pyrex tubes, 11 cm. long with internal diameters of 3 and 14 mm., and spun for an hour in an angle centrifuge at 3000 R.P.M. The supernatant fluid is saved for serological work and the sediment is resuspended in the same volume of buffer solution and spun for an hour in the angle centrifuge. The supernatant fluid is discarded and the sediment, resuspended in the same volume of buffer solution, is spun once more in the angle centrifuge for an hour at 3000 R.P.M. The supernatant fluid is again discarded and the sediment, resuspended in a quarter to a half of the original volume (20–25 cc.) of buffer solution, is spun for an hour in a horizontal centrifuge at 3000 R.P.M. The whitish, somewhat opalescent supernatant fluid which contains the washed elementary bodies is removed and used for studies by means of stains, titrations of infectivity, and serological reactions.

Sections for microscopic examination of skin removed on the 5th day after inoculation for the preparation of elementary bodies show (Figs. 1, 2) a marked thickening of the epithelial layer of cells; the number of cells is greater than that seen in normal skin, individual cells are larger than normal, the intercellular bridges are damaged or have disappeared, and many cells contain acidophilic cytoplasmic inclusions. The photographs demonstrate the superficial nature of the infection and indicate the reason why skin handled in the manner described is suitable for obtaining large amounts of elementary bodies of myxoma.

Four rabbits are usually handled in the same experiment in the manner described. In this way one obtains 25–50 cc. of a suspension of washed elementary bodies in a relatively pure state. When more than one rabbit is used, however, it should be remembered that the first part of the procedure, *i.e.*, the harvesting of the material from the rabbits and the collection of the first and second supernatant fluids by means of the horizontal centrifuge, must be carried out separately for each rabbit, after which all the supernatant fluids may be pooled before treatment in the angle centrifuge. This is necessary because the speed with which the coarse particles are removed from the suspensions seems to influence the number and the purity of the elementary bodies in the final preparations.

When smears from properly prepared suspensions are stained according to Morosow's (9) method, numerous round black bodies are seen (Fig. 3). Most of the bodies are discretely distributed but some occur in aggregations of two and three. A certain amount of brownish amorphous material, some of which is from the tannic acid used as a mordant, is observed in the spaces between the bodies.

Although repeated washing does not result in a suspension containing nothing but elementary bodies, the major portion of the particulate matter consists of them. Furthermore, the procedure insures the discarding or marked dilution of soluble components of the material harvested from the animals. In any event, a comparison of titers of the infectivity of suspension of washed bodies with those of suspensions before treatment in the angle centrifuge indicates that there is no great loss of infectivity due to the repeated washing. Three examples may be cited. In one, the suspension of unwashed

bodies (75 cc.) from 3 rabbits titered 10^{-5}, while the thrice washed bodies (35 cc.) just before the final spinning in the horizontal centrifuge titered 10^{-7}. In the second, the suspension of unwashed bodies (100 cc.) from 4 rabbits titered 10^{-8} and the thrice washed bodies (40 cc.) after being spun for an hour in the horizontal centrifuge titered 10^{-7}; a certain amount of the bodies is always thrown down and lost during the last treatment in the horizontal centrifuge. The third example is that in which the suspension of unwashed bodies (50 cc.) from 2 rabbits titered 10^{-7} while the thrice washed bodies (15 cc.) after an hour's spinning in the horizontal centrifuge titered 10^{-7}. Although the titers in this instance were the same, a number of bodies were lost as a result of the last spinning in the horizontal centrifuge; this fact becomes obvious when one realizes that the original suspension was in 50 cc. while the final one was in 15 cc., and that the lesions produced by the washed bodies were not so large as those caused by the untreated material.

Further evidence that the elementary bodies represent the virus of infectious myxomatosis or that the virus is intimately associated with them is obtained by a comparison of the infective titer of the supernatant fluid with that of the sediment made back to original volume after thorough sedimentation in an angle centrifuge. In such an experiment a suspension of elementary bodies that had been washed five times was spun in an air-driven angle centrifuge (10) for an hour at 14,000 R.P.M. Before centrifugation the suspension titered 10^{-4}; after centrifugation the supernatant fluid titered 10^{-1} while the titer of the sediment made back to volume was 10^{-4}. It is almost impossible to sterilize the supernatant fluid by ordinary centrifugation because the elementary bodies adhere to the walls of the tubes and become detached when the supernatant fluid is removed.

Vaccinal elementary bodies are harvested on the 3rd day after inoculation of the rabbits and a marked clumping of the bodies during the process of washing never occurs. Consequently, there is no great loss of the bodies during the last sedimentation in the horizontal centrifuge. The 5th day after inoculation of the animals appears to be the best time to harvest the elementary bodies of myxoma, and during the process of washing many of the bodies arrange themselves in clumps which are thrown down and lost during

tissue. Thereafter intradermal and subcutaneous inoculations of myxoma virus are continued for a period of 2 months, 2 cc. of a 10 per cent emulsion being administered at weekly or semiweekly intervals. At the end of this time 2 intraperitoneal injections, 1 cc. and 2 cc. respectively, of suspensions of elementary bodies, prepared according to the method described above and rendered free from viable bacteria by means of ether, are given; an interval of 4 days separates the 2 injections. Finally, 2 days after the last intraperitoneal inoculation, 2 cc. of a 10 per cent emulsion of infectious tissue are inoculated intradermally and subcutaneously. A week after the last inoculation the animals are bled and the serum is collected and inactivated.

Antifibroma Serum.—Just prior to the inoculation of myxoma virus and a month after the animals had been infected with fibroma virus, serum was collected from the rabbits for serological work.

Control Serum.—Serum from normal rabbits or from rabbits convalescent from vaccinal infections was used as a control.

Preparations of Materials for Precipitin Reactions.—Virus-free precipitinogens have been prepared from two sources. (*a*) It was stated above that the supernatant fluid resulting from the first sedimentation in the angle centrifuge of material harvested from the skin of an infected animal is saved for serological studies. This slightly infectious and somewhat cloudy fluid is rendered clear and free from virus by rapid passage through a Seitz filter through which 10 cc. of broth containing 10 per cent of normal rabbit serum has previously been filtered; the filtrates are tested for the presence of active virus by intradermal inoculation of rabbits. (*b*) Blood taken from rabbits 5 days after extensive inoculation of the scarified skin is always infectious; the serum collected from such blood after clotting has in our experience been free from demonstrable amounts of virus or has contained very little of the infectious agent. Nevertheless, in our work regarding the presence of soluble antigens in such serum we always filter it through a Seitz pad as described above, and then test for the presence of virus by inoculation of animals.

Agglutination Reactions.—The agglutination reactions are performed in the manner described by Craigie (3) and by Parker and Rivers (4). Different dilutions of the immune sera are made with physiological salt solution brought to pH 7.0 by the addition of 1 cc. of a 0.2 M citric acid-disodium phosphate buffer solution to each 100 cc. It is advisable to prepare the saline solution for each set of tests with recently boiled distilled water. The use of other diluents may cause a non-specific agglutination of the elementary bodies. 0.25 cc. of each dilution of serum is placed in pyrex test tubes with outside dimensions of 10 x 75 mm. To each tube is then added 0.25 cc. of a suspension of washed elementary bodies. Light suspensions of bodies yield the best results, the proper density being learned by experience. Controls are included in every experiment. The tubes are covered in a manner (12) to prevent evaporation and are placed in an incubator at 50°C. for 18–20 hours.

Precipitin Reactions.—Usually the proper dilution of precipitinogen for pre-

TABLE 1

Summary of Results of Agglutination Reactions Conducted with Antimyxoma Serum, Normal Serum, and Three Preparations of Myxoma Elementary Bodies

					Dilution of serum						
Antimyxoma serum plus	Undiluted	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Myxoma E.B. 7481	++	+++	++++	++++	++++	++++	++++	++++	+	±	-
" " 7515	++	+++	++++	++++	++++	++++	++++	++	±	-	-
" " 7515 (T)	+	++	+++	++++	++++	++++	+	-	-	-	-
Normal serum plus											
Myxoma E.B. 7481	+±	++	++	±	-	-	-	-	-	-	-
" " 7515	+	±	±	±	±	-	-	-	-	-	-
" " 7515 (T)	-	±	±	±	±	-	-	-	-	-	-

Pluses indicate degree of agglutination. Controls of elementary body suspensions and sera were negative. E.B. = elementary bodies. E.B. suspensions 7481 and 7515 were prepared according to routine. E.B. suspension 7515 (T) was prepared, with the aid of trypsin, from the elementary bodies lost during the last horizontal centrifugation of suspension 7515. For the agglutination reactions, the suspensions were diluted in such a manner that they contained approximately the same number of elementary bodies.

cipitation reactions is determined by preliminary titrations (12). For the type of work described at the present time this is not essential. Dermal filtrates or sera that are being tested for precipitinogens are arbitrarily used undiluted and diluted 1:2 and 1:4. Different dilutions of immune serum and control serum are made and distributed in pyrex tubes as described for agglutination reactions. To the material in each tube is added an equal amount of the dermal filtrate or serum which is being tested for the presence of precipitinogens. At times varying dilutions of the material being tested for precipitinogens are used, and to them are added equal amounts of the immune or control serum diluted 1:2, 1:4, or 1:8. The mixtures, protected against evaporation, are incubated at 50°C. for 18-20 hours.

TABLE II

Summary of Results of Agglutination Reactions Conducted with Myxoma Elementary Bodies and Antimyxoma Serum, Antivaccinal Serum, Antifibroma Serum, and Normal Serum, Respectively

Myxoma elementary bodies (7481) plus	Dilution of serum								
	Undi-luted	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Antimyxoma serum..................	+	++	++	++	++	++	++	++	+
Antifibroma serum..................	+	+	++	++	++	+	±	−	−
Antivaccinal serum.................	±	−	−	−	−	−	−	−	−
Normal serum.....................	+	+	−	−	−	−	−	−	−

Pluses indicate degree of agglutination. Controls of elementary bodies and the different sera were negative.

A number of agglutination and precipitation tests have been conducted; the former were made with washed elementary bodies prepared with and without trypsin, while the latter were done with dermal filtrates and filtered serum from animals acutely ill with myxoma. The materials used for the precipitation tests contained no elementary bodies and were not infective. Serum from animals recovering from fibroma, hyperimmune antimyxoma serum prepared in rabbits immune to fibroma, hyperimmune antivaccinal serum, and normal serum were used. An examination of the results, some of which are summarized in Tables I to V, indicates (*a*) that the elementary bodies of myxoma are specifically agglutinated by antimyxoma serum, (*b*) that a certain amount of agglutination of myxoma elementary bodies occurs in the presence of convalescent fibroma

TABLE III

Summary of Results of Precipitation Reactions Conducted with Two Dilutions of a Myxoma Dermal Filtrate and Antimyxoma Serum, Antivaccinal Serum, and Normal Serum, Respectively

Antimyxoma serum plus	Dilution of serum								
	Undi-luted	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Myxoma filtrate 1:2	+	++	++	++	+	+	−	−	−
" " 1:4	±	++	++	++	+	+	±	−	−
Antivaccinal serum plus									
Myxoma filtrate 1:2	−	−	−	−	−	−	−	−	−
" " 1:4	−	−	−	−	−	−	−	−	−
Normal serum plus									
Myxoma filtrate 1:2	−	−	−	−	−	−	−	−	−
" " 1:4	−	−	−	−	−	−	−	−	−

Pluses indicate degree of precipitation. The dermal filtrate was prepared in the manner described in the text and was not infective.

TABLE IV

Summary of Results of Precipitation Reactions Conducted with Two Dilutions of a Myxoma Dermal Filtrate and Antimyxoma Serum, Antifibroma Serum, and Normal Serum, Respectively

Antimyxoma serum plus	Dilution of serum								
	Undi-luted	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Myxoma filtrate 1:2	±	++	++	++	+	+	±	−	−
" " 1:4	±	++	++	+	+	+	±	−	−
Antifibroma serum plus									
Myxoma filtrate 1:2	±	+	+	±	−	−	−	−	−
" " 1:4	±	+	±	±	−	−	−	−	−
Normal serum plus									
Myxoma filtrate 1:2	−	−	−	−	−	−	−	−	−
" " 1:4	−	−	−	−	−	−	−	±	−

Pluses indicate degree of precipitation. The dermal filtrate was prepared in the manner described in the text and was not infective.

serum, a result to be expected in view of previous work of Shope (11), Berry and Dedrick (5), Berry and Lichty (13), and Ledingham (7), (*c*) that a specific precipitate occurs when a myxoma virus-free dermal filtrate is mixed with antimyxoma serum, (*d*) that a mixture of serum from a rabbit convalescent from fibroma with a myxoma virus-free dermal filtrate results in a precipitation less marked than the one just described, and (*e*) that in the serum of an animal acutely ill with myxoma a specific non-infectious precipitinogen occurs. There

TABLE V

Summary of Results of Precipitation Reactions Conducted with Mixtures of Normal Serum, Antimyxoma Serum, and Antivaccinal Serum, Respectively, with Two Dilutions of Serum Collected from Rabbits Acutely Ill with Myxoma

Antimyxoma serum plus	Dilution of serum								
	Undiluted	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Myxoma serum undiluted.............	++	++	++	++	++	±	−	−	−
Myxoma serum diluted 1:2...........	+	++	+	+	+	+	−	−	−
Antivaccinal serum plus									
Myxoma serum undiluted.............	−	−	−	−	−	−	−	−	−
Myxoma serum diluted 1:2...........	−	−	−	−	−	−	−	−	−
Normal serum plus									
Myxoma serum undiluted.............	−	−	−	−−	−	−	−	−	−
Myxoma serum diluted 1:2...........	−	−	−	−	−	−	−	−	−

Pluses indicate degree of precipitation. The serum from myxomatous rabbits containing the precipitinogen was collected and prepared according to the manner described in the text.

are some indications that elementary bodies prepared with trypsin are more likely to undergo spontaneous agglutination and are less agglutinable by specific serum than are bodies handled according to routine. It is not clear whether these differences are due to the use of trypsin or to the additional washings incident to the use of trypsin.

COMMENTS AND SUMMARY

From the results of the experiments described in this paper it is obvious that large amounts of elementary bodies of myxoma can be

obtained in a relatively pure state by means of the methods used. Furthermore, it is evident that infectious myxomatosis is a viral disease in which elementary bodies of the same order of magnitude as vaccinal elementary bodies play a conspicuous rôle in that they either represent the etiological agent or are intimately associated with it. The bodies are specifically agglutinated by antimyxoma serum and are agglutinated to a less extent by serum from rabbits convalescing from fibroma, a disease closely related to myxoma. In virus-free filtrates of emulsions prepared from infected skin there is a soluble precipitinogen or precipitinogens specific for the malady. Moreover, a specific precipitinogen or precipitinogens are demonstrable in virus-free serum of animals acutely ill as a result of extensive infection with myxoma virus. It is believed that this is the second viral disease, yellow fever (14) being the first, in which a specific soluble antigen free from virus has been found in the serum of ill animals.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Photograph of section of skin infected with myxoma virus. The inoculation was made in manner described in text and the skin was removed 5 days later. Marked hyperplasia of the epithelial cells has occurred; many of the cells contain cytoplasmic inclusions and are beginning to undergo disintegration. Hematoxylin and eosin. × 400.

FIG. 2. High magnification of epithelial cells infected with myxoma virus showing cytoplasmic inclusions. Hematoxylin and eosin. × 1000.

FIG. 3. Washed elementary bodies of myxoma prepared according to routine. Morosow's stain. × 1000.

FIG. 4. Washed elementary bodies of myxoma salvaged by means of trypsin from the sediment resulting from the last horizontal centrifugation in the routine method described in the text. Morosow's stain. × 1000.

FIG. 5. Washed elementary bodies prepared according to routine except that clumping was prevented by the addition of a small amount of trypsin to the material harvested from rabbits prior to its treatment in the angle centrifuge. Morosow's stain. × 1000.

FIG. 6. Sediment consisting of clumped elementary bodies resulting from the last horizontal centrifugation in the routine method of preparation of such bodies described in the text. Morosow's stain. × 1000.

FIG. 7. To the sediment, a sample of which is shown in Fig. 6, a small amount of trypsin was added. Immediately the clumps were disrupted and the elementary bodies became discretely distributed as depicted. Morosow's stain. × 1000.

PLATE 2
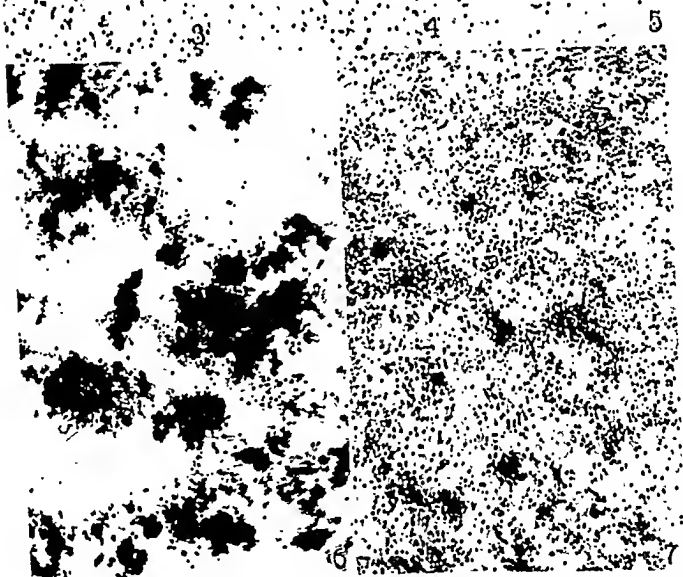
(Rivers and Ward· Elementary bodies of myxoma)

INFLUENCE OF HOST FACTORS ON NEUROINVASIVENESS OF VESICULAR STOMATITIS VIRUS

I. Effect of Age on the Invasion of the Brain by Virus Instilled in the Nose*

By ALBERT B. SABIN, M.D., and PETER K. OLITSKY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

An unusual type of acquired resistance of growing animals to infection with certain neurotropic viruses was described in a previous communication (1). This resistance was associated neither with prior exposure to infection, nor with antiviral bodies in the blood. Moreover, it was demonstrable when the virus was injected by peripheral routes but not when inoculated directly into the brain. It appeared, therefore, that certain changes in the host, associated with or the result of the process of growth, could either modify or completely suppress the effects of peripherally inoculated virus. The purpose of the present investigation was to determine the mechanism of this resistance and to ascertain as far as possible the nature of the host changes responsible for it.

Methods

For an analysis of the factors underlying the resistance of older animals to peripheral inoculation of the neurotropic viruses under investigation, it was necessary to know in each instance how the virus acted in animals succumbing to infection, *i.e.*, (a) whether or not a primary phase of systemic infection was induced; (b) from which tissues and by what routes it spread to the central nervous system (C.N.S.); and (c) what course it pursued after the latter had been

invaded. The primary objective, therefore, was to obtain precise data on the pathogenesis of the experimental disease in susceptible animals, and at the same time to determine the variations associated with natural resistance.

Virus and Animals.—The present study was carried out with the immunologically distinct New Jersey and Indiana strains of vesicular stomatitis virus in Rockefeller Institute albino mice of known ages. Unless otherwise indicated, freshly passaged virus was used. The brains of two or three mice succumbing
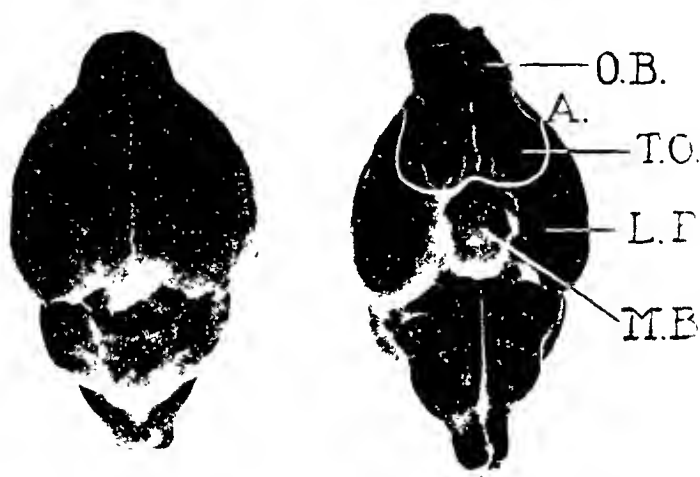
Fig. 1. Dorsal and ventral aspects of mouse brain. O.B., olfactory bulb; T.O., tuberculum olfactorium; L.P., lobus pyriformis; M.B., mammillary body; line A, line along which the brain was sectioned into anterior rhinencephalon and remainder of the brain.

after intracerebral injection were ground with alundum and sufficient hormone broth to make a 10 per cent suspension. The supernatant fluid obtained after horizontal centrifugation at about 2,000 R.P.M. for 10 minutes was designated as the 1:10 dilution of virus. The virus suspension was titrated in each experiment to establish the approximate number of minimal cerebral lethal doses (M.C.L.D.) which were inoculated into animals. Dilutions of 10^{-5}, 10^{-6}, and 10^{-7} were given intracerebrally to groups of two or three mice for each dilution (0.03 cc. for each mouse). Death within 10 days of one animal with characteristic signs of encephalitis established the infectivity of the dilution employed.

Tests for Virus in Inoculated Animals.—Mice sacrificed at various intervals after inoculation were anesthetized and bled to death by cardiac puncture. Blood to be examined for virus content was drawn into a sterile syringe containing heparin. The presence of virus in various tissues was determined by grinding them in broth, usually without alundum, and by injecting 0.03 cc. of the whole suspensions, or their dilutions, into the brain of each of two or three mice (3 to 4 weeks old animals were used in tests for virus and for titrations). Mice dying within 24 hours were discarded, since death proved to be due to injury or bacterial contamination. When mice became ill or died within 10 days, film preparations of their brains were examined for organisms; if none was seen, passage was frequently made to establish that the virus was the cause of death. Signs of encephalomyelitis developing later than 10 days, with one exception, proved not to be due to vesicular stomatitis virus. In three different experiments encephalomyelitis occurred 15 to 17 days after intracerebral inoculation of mouse blood in one instance and of mouse brain in the other two. A virus was isolated from the first mouse, which after an intensive study proved to be similar to the virus of mouse spontaneous encephalitis described by Theiler (2). In two other experiments animals developed marked circling and ataxia about 15 days after inoculation; neither bacteria nor virus was recovered from them.

Methods of Examination of Nervous Tissues.—At this point it should be recalled that the gross anatomy of the brain of the mouse varies somewhat from that of the rat, guinea pig, and rabbit in the prominence of certain important landmarks. In the mouse the rhinal fissure is very faint, in places even indiscernible in fresh specimens, and extends rather more dorsally, so that the entire ventral and ventrolateral aspects of the cortex to either side of the diencephalic and mesencephalic structures and caudad to the optic chiasm constitute the pyriform lobes (Fig. 1). Just anterior to the optic chiasm are the olfactory tubercles, represented by two protuberances which are much more prominent than in the other rodents.

Since different parts of the brain were separately studied for virus content, care was taken to avoid contamination of one part with another when removing the organ from the cranial cavity. Separate sterile instruments were used in obtaining particular structures.

After nasal instillation, the brain was usually separated into two parts: it was cut at the level of the optic chiasm (line A, Fig. 1), obliquely, anteriorly, and ventrodorsally. The anterior portion, hereafter referred to as "anterior rhinencephalon," thus comprised chiefly the olfactory bulbs, tracts, and tubercles, portions of the remaining rhinencephalic cortex anterior to the optic chiasm, and parts of the corpus striatum. The posterior portion, designated as "remainder of the brain," was made up of what was left of the brain, including the cerebellum, pons, and medulla.

Test for Virus in Nasal Mucosa.—The skin was reflected aseptically and the entire nasal contents, mucosa, cartilage, and bone, were scraped out, taking care to leave the cribriform plate intact to avoid inclusion of olfactory bulb material. The scrapings were ground with alundum and 2 cc. of hormone broth, and centrifuged horizontally at about 2,000 R.P.M. for 45 minutes to sediment most of the

bacteria. The supernatant liquid and dilutions thereof in broth could be injected intracerebrally in mice without inducing bacterial meningitis.

EXPERIMENTAL

Distribution of Virus in Young Mice Succumbing to Infection by Cerebral or Nasal Routes.—The purpose of this experiment was to disclose any evidence of a generalized infection at the termination of the disease in young mice given the virus intracerebrally[1] or intranasally.

TABLE I

Distribution of Vesicular Stomatitis Virus in Various Tissues of Mice Succumbing to Intranasal or Intracerebral Inoculation

Tissue tested for virus	From mice succumbing after	
	Nasal instillation of V.S.-N.J.	Intracerebral inoculation of V.S.-Ind.
Blood...................................	0, 0	0, 0
Lungs...................................	0, 0	0, 0
Spleen...................................	0, 0	0, 0
Liver...................................	0, 0	0, 0
Suprarenals...................................	0, 0	n. t.
Sciatic nerves...................................	0, 0	"
Brain...................................	2, 2	2, 2

V.S.-N.J., V.S.-Ind. = vesicular stomatitis virus, New Jersey or Indiana strains.

n. t. = no test was made.

Numbers indicate the day of death of individual mice, 0 signifying survival.

15 day old mice were inoculated intracerebrally or intranasally with the New Jersey or Indiana strains of virus. Mice receiving virus in the brain died within 2 days, and in the nose, within 5 days. When prostrate, they were exsanguinated. The blood, lungs, spleen, liver, suprarenals, sciatic nerves, and brain from each of two mice were pooled and tested for virus. Approximately 10 per cent suspensions of the organs in broth were used for injections into the brain.

The results (Table I) indicate that among the tissues examined, only the brain contained virus. While this experiment does not entirely eliminate the possibility of a mild infection of some of the organs at an earlier stage of the disease, particularly in the nasally

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[1] All such operations were done with the aid of ether anesthesia.

infected mice in which the disease lasted for 5 days, it offers no evidence that generalized infection occurred. In the intracerebrally injected mice, in which the disease lasted only 2 days, there is little doubt that neither the spleen, liver, nor lung was attacked by the virus.

Virus in Various Tissues of Young and Old Mice at Intervals after Nasal Instillation.—The present experiment was designed to show (a) whether virus was demonstrable in the blood at any time after nasal instillation of relatively large amounts (10^4 to 10^5 M.C.L.D.); (b) whether it multiplied at any time in the lung or spleen; (c) how soon it could be revealed in the C.N.S.; and (d) how the results obtained in the uniformly susceptible, 15 day old mice compared with those in 1 year old animals.

15 day and 1 year old mice were given intranasally 0.03 cc. of 1:100 dilution of virus (10^5 M.C.L.D., N. J. strain). At intervals indicated in Table II, two young and two old animals were sacrificed. The blood or organ suspensions of the two mice from each group were pooled and 0.03 cc. injected intracerebrally into each of three test mice. On the 4th day after nasal instillation, the young animals showed signs of C.N.S. involvement and all of them were dead on the next day, while all the old ones remained well during the 6 days of the test.

The experiment recorded in Table II presents the results of tests for virus in the blood, lung, spleen, and the olfactory brain anterior to the optic chiasm including the bulbs. Neither the young nor old mice exhibited virus in the blood examined at 3 to 5 minutes, 5 and 24 hours, 2, 4, and 6 days after instillation. Nor was virus found in the spleens of animals of both groups, on the 2nd, 4th, and 6th days. The lungs of old mice had no demonstrable virus, while a small amount was detected in these organs of the young in the 2nd, and none on the 4th day. Relatively large amounts (as expressed by the short incubation period and death of all the test mice) were present in the anterior rhinencephalon of the young on the 2nd and 4th days. The striking finding was the recovery of virus from the anterior olfactory region of the old mice, apparently in small amount on the 2nd, and in larger quantity on the 4th and 6th days.

These results reveal again that no generalized systemic infection follows nasal instillation of the virus in either young or old mice, and that the C.N.S. is invaded in old mice, as well as in young animals that readily succumb to infection.

Extent of Virus Invasion in the Central Nervous System of Young and Old Mice after Nasal Instillation.—In the preceding experiment in which only the rhinencephalon anterior to the optic chiasm was tested, it was disclosed that virus was present in this region in both young and old mice, although in apparently greater concentration in the

TABLE II

Invasion of Various Tissues of Young and Old Mice at Different Intervals after Nasal Instillation of New Jersey Vesicular Stomatitis Virus

Tissue tested for virus*	Time after nasal instillation	Age of mice supplying tissues	
		15 days Average weight, 6.5 gm.	About 1 yr. Average weight, 35 gm.
Heparinized blood	3–5 min.	0, 0, 0	0, 0, 0
	5 hrs.	0, 0, 0	0, 0, 0
	24 "	0, 0, 0	0, 0, 0
	2 days	0, 0, 0	0, 0, 0
	4 "	0, 0, 0	0, 0, 0
	6 "	†	0, 0, 0
Lungs	2 "	3, 6, 0	0, 0, 0
	4 "	0, 0, 0	0, 0, 0
	6 "	†	0, 0, 0
Spleen	2 "	0, 0, 0	0, 0, 0
	4 "	0, 0, 0	0, 0, 0
	6 "	†	0, 0, 0
Anterior rhinencephalon	2 "	2, 2, 2	8, 0, 0
	4 "	2, 2, 2	2, 2, 8
	6 "	†	4, 4, 0

Figures as in Table I.

* Tissues from two mice were pooled for these tests.

† Young mice were either dead or prostate from virus infection on 5th day, so that none was examined for virus later than 5 days.

former. It was pointed out in the previous communication (1) that while most 1 year old mice are resistant to nasal instillation of the vesicular stomatitis virus, a certain number develop encephalomyelitis 8 to 14 days after inoculation, and that of these some survive with persistent paralysis or other signs of encephalitis. The number whose

resistance is reflected only in a delayed incubation period or survival has been found to vary in different groups of mice and to depend to a degree upon the quantity of virus instilled intranasally. Thus, while a 10^{-3} or 10^{-4} dilution (about 10^3 or 10^2 M.C.L.D.) given by way of the nose is fatal for almost all 15 day old mice, it proved exceptional for one out of a group of ten 1 year old animals to succumb. When 10^5 or more M.C.L.D. of virus are instilled, the number of old mice which develop encephalomyelitis increases. In different experiments the number varied from one to five mice out of groups of ten. It may be mentioned here that resistance to intramuscular injection is much more complete in that not a single old mouse of the many inoculated thus far has shown any signs of disease, even after inoculations of 10 million cerebral lethal doses.

For a study of the fate of the virus in the C.N.S. of resistant old mice inoculated intranasally, it was necessary therefore to study material from individual mice rather than the pooled specimens from several animals, and also to investigate the course of events with a dose of virus which is almost constantly fatal for 15 day old, and almost constantly ineffective in 1 year old mice. In view of the suggestion in the preceding experiment that the brain of resistant mice may be invaded by virus, it was desirable to determine whether it was present in the entire C.N.S. or limited to certain regions only.

To investigate the latter point it was proposed to test for virus in two parts of the brain: (a) the rhinencephalic cortex (including part of the corpus striatum) anterior to the optic chiasm, and (b) the remainder of the brain including the cerebellum, pons, and medulla.

15 day and 1 year old mice which received intranasally 0.03 cc. of 10^{-2} dilution of the N. J. strain of virus (10^4 to 10^5 M.C.L.D.) were sacrificed at the following intervals after instillation: 3 to 5 minutes, 5 hours, and 2, 3, 5, 7, 10, and 16 days. All the young mice showed signs of encephalitis on the 4th, and were either dead or prostrate on the 5th day, so that none of them was examined for the presence of virus later than 5 days. None of the 1 year old mice which were sacrificed for these tests exhibited any signs of disease.

Another group of 15 day old and 1 year old mice was given 0.03 cc. of 10^{-3} dilution of the same virus suspension (10^3 to 10^4 M.C.L.D.) in the nares, and individuals were sacrificed at 2, 3, 5, 7, and 10 day intervals. Here again the young succumbed on the 5th day, while none of the old animals showed signs of disease.

The anterior rhinencephalon was ground in 0.3 cc. and the remainder of the

brain in 1 cc. of broth.   Good emulsification of tissue was secured without the use of an abrasive and centrifugation was not needed.   0.03 cc. of the tissue suspension was injected intracerebrally into each of three mice, so that with the material derived from the anterior rhinencephalon suspended in 0.3 cc., it was expected that the virus could be detected when 10 M.C.L.D., or perhaps even less, were present.   The tissue comprising the remainder of the brain, on the other hand, would necessarily have to contain 30 M.C.L.D. or thereabout before virus might be demonstrable.

The results shown in Table III indicate that after nasal instillation of 10^4 to 10^5 M.C.L.D., no virus was demonstrable in the brains of young or old mice at 3 to 5 minutes, or at 5 hours.   2 days after instillation the anterior rhinencephalon of the 15 day old animal yielded abundant virus, while the remainder of the brain had but a trace, *i.e.*, enough to induce fatal infection in only one of the three mice injected.   On the 3rd and 5th days, apparently large amounts of virus were found in both parts of the brain of the young animals.   A somewhat different course of events appears to have taken place in the old mice which were given the same amount of virus intranasally.   Again no virus was demonstrable in either portion of the brain at 3 to 5 minutes, or at 5 hours.   On the 2nd and 3rd days it was easily detectable in the anterior rhinencephalon but not at all in the remainder of the brain.   On the 5th day virus was present in the anterior rhinencephalon and probably in somewhat greater concentration than before (as evidenced by the shorter incubation period in all three test mice), while only a trace was found in the remainder of the brain (only one of the three test mice succumbing).   On the 7th day one mouse showed no virus in either part of the brain, while in another animal it was present in both parts.   No virus was demonstrable in the brains of old mice 10 and 16 days after instillation.

The results obtained with the group of animals which was instilled with one-tenth the amount of virus (10^3 to 10^4 M.C.L.D.) were similar and more clear cut.   In the 15 day old mice abundant virus was present in the anterior rhinencephalon on the 2nd day, while none was demonstrable in the rest of the brain.   On the 3rd and 5th days both parts of the brain showed considerable amounts of virus. Among the 1 year old animals, the anterior rhinencephalon of the 2nd day mouse contained a trace of virus, none was recovered in the 3rd day animal, and enough virus to kill all three mice after short incubation periods was present in the 5th day animal.   In none of these was virus detected in the rest of the brain.   In the 7th and 10th day mice no virus was found in either part of the brain.

From a review of these results, it appears that after nasal instillation in young mice, the virus first invades and multiplies somewhere in the anterior rhinencephalon and only after a certain interval spreads to the remainder of the brain, where it undergoes further multiplication until the animal dies.   In old mice the virus also invades the anterior rhinencephalon and apparently multiplies in it up until about the 5th

TABLE III

Extent of Invasion of Virus in Central Nervous Systems of Young and Old Mice at Various Intervals after Its Nasal Instillation

Amount of virus given intranasally	Part of brain tested for virus	Time after nasal instillation	Age of mice	
			15 days	About 1 yr.
0.03 cc. of 10⁻² mouse brain suspension in broth (10⁴ to 10⁵ M.C.L.D.)	Anterior rhin-encephalon	3–5 min.	0, 0, 0	0, 0, 0
		5 hrs.	0, 0, 0	0, 0, 0
		2 days	2, 2, 2	3, 4, 5
		3 "	2, 3, 3	2, 3, 8
		5 "	2, 2, 2	2, 2, 3
		7 "	*	† {0, 0, 0 / 2, 2, 3}
		10 "	*	0, 0, 0
		16 "	*	0, 0, 0
	Remainder of brain	3–5 min.	0, 0, 0	0, 0, 0
		5 hrs.	0, 0, 0	0, 0, 0
		2 days	3, 0, 0	0, 0, 0
		3 "	2, 3, 4	0, 0, 0
		5 "	2, 2, 2	5, 0, 0
		7 "	*	† {0, 0, 0 / 2, 4, 5}
		10 "	*	0, 0, 0
		16 "	*	0, 0, 0
0.03 cc. of 10⁻⁵ of same suspension (10² to 10⁴ M.C.L.D.)	Anterior rhin-encephalon	2 days	3, 3, 3	4, 0, 0
		3 "	2, 2, 2	0, 0, 0
		5 "	2, 2, 2	3, 4, 4
		7 "	*	† {0, 0, 0 / 0, 0, 0}
		10 "	*	† {0, 0, 0 / 0, 0, 0}
	Remainder of brain	2 "	0, 0, 0	0, 0, 0
		3 "	2, 2, 2	0, 0, 0
		5 "	2, 2, 2	0, 0, 0
		7 "	*	† {0, 0, 0 / 0, 0, 0}
		10 "	*	† {0, 0, 0 / 0, 0, 0}

Figures and asterisks same as in Tables I and II.

† Indicates that two animals were tested for virus on the day mentioned.

day. In completely resistant animals which show no signs of disease, it either does not spread beyond the anterior rhinencephalon or if it does, fails to multiply in the remainder of the brain. In the partly resistant old mice, *i.e.* those which exhibit signs of encephalomyelitis after some delay, the virus reaches, and multiplies in the remainder of the brain, but the interval between infection of the anterior rhinencephalon and involvement of the remaining cerebral tissue appears to be much longer.

In the light of the present findings, it is important to recall that after intracerebral injection of this virus, young and old mice are equally susceptible to infection. Thus, while direct inoculation into the brain of a minimal amount of virus is fatal in old mice, larger amounts may be present in the anterior rhinencephalon for a number of days after nasal instillation, without involving those parts of the C.N.S., damage of which leads to signs of encephalomyelitis and to death. It seemed possible, therefore, that nasally instilled and intracerebrally injected virus may, perhaps, be distributed by different pathways within the C.N.S.

Spread of Intracerebrally Injected Virus in the Central Nervous System of Old Mice.—A consideration of the different behavior of intracerebrally injected and intranasally instilled virus in old mice suggests that the former might have spread to various parts of the brain by way of the cerebrospinal fluid in the subarachnoid space or ventricles, while the latter might have traveled in a closed system along specific tracts. The apparent halting of the progression of the virus at certain points in the brain of intranasally instilled animals might thus be due to the presence of certain barriers, perhaps in the form of localized zones of insusceptibility. If virus placed in the brain spread by the way of the spinal fluid, it was believed that within a short time after inoculation into one side of the brain it should be demonstrable in the opposite side.

To test this point, two groups of 1 year old mice were injected intracerebrally well to the right of the dorsal midline and approximately in the parietal region. One group received 0.03 cc. of 10^{-5} dilution of virus (10^1 to 10^2 M.C.L.D.) and the other the 10^{-4} dilution of the same material (10^2 to 10^3 M.C.L.D.). One mouse from each group was exsanguinated at 45 minutes and another at 24 hours after inoculation. The dorsal and lumbar cord were first removed and then the entire brain.

The cerebellum, pons, and medulla having been cut away, the brain was divided in the midline along the longitudinal fissure and the right and left hemispheres examined separately for virus. Each hemisphere was ground in 1 cc. of broth, while the dorsal and lumbar cord, in 0.5 cc. Of each suspension 0.03 cc. was introduced into the brain of each of three test mice.

The probability of recovering virus from one of the hemispheres depended, under the conditions of this experiment, on its content of at least 10 to 30 M.C.L.D. Consequently it was not surprising to find that no virus was detected at 45 minutes in any part of the C.N.S. of the mouse given 10 to 100 M.C.L.D., but rather unexpected was the fact that this should also obtain for the animal receiving 10^2 to 10^3 M.C.L.D. (Table IV). It appeared likely that a considerable fraction of the injected virus had either been removed or rendered non-infective within 45 minutes

TABLE IV

Spread of Virus in Central Nervous System of Old Mice after Intracerebral Injection

Amount of virus injected intra-cerebrally on right side	Time after inoculation	Presence of virus in central nervous system			
		Right hemisphere	Left hemisphere	Cerebellum	Dorsal and lumbar cord
	hrs.				
0.03 cc. of 10^{-6} (10^1 to 10^2 M.C.L.D.)	0.75	0, 0, 0	0, 0, 0	n. t.	0, 0, 0
	24.0	2, 2, 4	3, 3, 3	"	3, 3, 4
0.03 cc. of 10^{-4} (10^2 to 10^3 M.C.L.D.)	0.75	0, 0, 0	0, 0, 0	"	0, 0, 0
	24.0	2, 3, 3	2, 3, 3	"	0, 0, 0
0.01 cc. of 10^{-1} (10^5 to 10^6 M.C.L.D.)	1.5	3, 4, 5	4, 5, 5	3, 5, 0	0, 0, 0
	24.0	2, 2, 3	2, 4, 4	3, 4, 4	2, 2, 4

Abbreviations as in Table I.

after inoculation, although a certain amount of it, probably insufficient for detection, must have remained. In the mice examined at 24 hours, both hemispheres, and in one instance the lower cord as well, contained abundant virus. The results failed, however, to answer satisfactorily the question of this experiment, since widespread distribution of virus at 24 hours is in itself no indication of spinal fluid spread.

Another group of 1 year old mice was therefore injected in the same way but with more virus (10^5 to 10^6 M.C.L.D.). Mice were etherized at 1½ and 24 hours and in addition to the tissues previously tested for the presence of virus, the cerebellum was also included. The results (Table IV) reveal that in this test virus was demonstrable within 1½ hours in both hemispheres and cerebellum, but not in the cord, while at 24 hours all tissues examined contained considerable quantities.

The data obtained in these experiments show that after intracerebral injection, virus is rapidly distributed to remote parts of the brain, and even when the amount originally deposited in a certain area is too small for detection by the methods here employed, it readily multiplies within less than 24 hours.

The observation made in the earlier experiments that after nasal instillation appreciable amounts of virus are present in the anterior rhinencephalon for at least 2 or 3 days, without involving the remainder of the brain, becomes more significant. The fact that even a minimal amount of virus given intracerebrally becomes readily detectable, even in remote parts of the brain, within 24 hours suggests that in the old mice which are resistant to nasal instillation the virus is arrested in its progression somewhere in the anterior rhinencephalon, where some sort of effective barrier therefore exists.

Local Multiplication of Virus after Nasal Instillation in Young and Old Mice.—In view of the different behavior of this virus within the C.N.S. of young and old mice, it was of interest to determine whether or not the age of the host exerted any influence on the fate of virus deposited on the nasal mucosa, particularly since its olfactory portion is the seat of many nerve cells, the olfactory neurones of the first order.

One group of 15 day old mice and another of animals about 1 year old were instilled nasally with virus (N.J.), each receiving approximately 10^5 M.C.L.D. in 0.03 cc. of broth. At intervals of 1 hour and 2, 4, and 5 days, mice were exsanguinated; the entire mucosa was scraped out and prepared as already described under Methods. The undiluted centrifuged, supernatant liquid and tenfold dilutions of it in broth were inoculated intracerebrally into each of two mice. Since a variable number of old mice succumb with encephalitis after nasal instillation of virus, it appeared advisable to test separately the nasal mucosa of a number of animals to disclose any individual variation.

The data summarized in Table V reveal significant although unexpected results. In both young and old mice sacrificed 1 hour after nasal instillation of approximately 10^5 M.C.L.D., no virus was demonstrable in the nasal mucosa. Considering the total volume (2 cc.) of fluid in which the tissue of each mouse was suspended and the amount (0.03 cc.) which constituted the test dose for intracerebral inoculation, it is clear that approximately 66 M.C.L.D. would have to be present in the suspension before virus could be demonstrated. It is

plain, nevertheless, that, with the possible exception of a very small amount of virus (not demonstrable in this instance), most of the original 10^5 M.C.L.D. disappeared from the nasal mucosa shortly after instillation. 2 and 4 days later, however, the mucosa of young mice contained sufficient virus, detectable even in 10^{-3} dilution of the centrifuged suspension.

In old mice, on the other hand, virus was recovered in low concentration (1:10 of centrifuged suspension) from the nasal mucosa in only

TABLE V

Presence of Virus in Nasal Mucosa of Young and Old Mice at Varying Intervals after Nasal Instillation of 10^5 M.C.L.D.

Age of mice supplying nasal mucosa	Time after nasal instillation	Mouse	Test of virus in centrifuged suspension of nasal mucosa			
			Undiluted	1:10	1:100	1:1,000
15 days	1 hr.	1	0, 0	0, 0	0, 0	n. t.
	2 days	2	2, 2	3, 3	0, 0	3, 0
	4 "	3	3, 4	3, 3	3, 3	6, 0
About 1 yr.	1 hr.	1	0, 0	0, 0	0, 0	n. t.
		2	0, 0	0, 0	n. t.	"
		3	0, 0	0, 0	"	"
	2 days	4	0, 0	0, 0	0, 0	0, 0
		5	0, 0	0, 0	0, 0	0, 0
		6	2, 3	3, 4	0, 0	0, 0
	5 "	7	0, 0	0, 0	0, 0	0, 0
		8	4, 6	4, 4	0, 0	0, 0

Abbreviations as in Table I.

one of three animals sacrificed on the 2nd and one of two on the 5th day. The failure to demonstrate virus in this tissue of many old mice at a time when it is readily detectable in the anterior rhinencephalon suggests that the C.N.S. may be invaded with no, or very slight and transitory local multiplication.

Factors Concerned in Arresting Virus Progression in Brains of Old Mice.—The apparent failure of virus to progress beyond a certain region in the brain may be due to a number of elements, but first it is

essential to establish, as well as can be, whether these factors exist in the animal before experimental infection is initiated, or whether they are the result of a rapidly acquired, specific immunity.   If the arrest of virus progression were due to a preexisting, localized barrier, the intracerebral inoculation of a small amount of virus, at a time when the anterior rhinencephalon is known to contain readily detectable amounts of the infective agent, should be capable of circumventing such a barrier and inducing a lethal infection.   If, however, the further progression of virus is inhibited by the development of specific tissue immunity in the rest of the brain, such an inoculation into mice, previously given virus by way of the nose, should prove innocuous.

TABLE VI

Is the Arrest of Virus Spread in the Brains of Nasally Instilled Old Mice the Result of Preexisting Conditions or of a Rapidly Acquired Specific Immunity?

History of mice	No. of mice injected	Effect of intracerebral injection of 0.3 cc. of 10^{-5} suspension of glycerolated virus (about 1 M.C.L.D.)
Old mice 2 days after nasal instillation of 10^{-2} virus suspension (N. J.) (10^4 to 10^5 M.C.L.D.)	6	* 5, 5, 8, 8, 0, 0
Normal old mice, controls	6	4, 4, 5, 7, 0, 0
Old mice 4 days after nasal instillation of 10^{-2} virus suspension (10^4 to 10^5 M.C.L.D.)	6	4, 4, 4, 5, 5, 0
Normal old mice, controls	6	4, 5, 5, 0, 0, 0

* The numbers indicate day of death after intracerebral inoculation.

Two experiments were performed: one employing the N. J. strain and another, somewhat modified, the Indiana strain.   In the first experiment (Table VI) twelve 1 year old mice were each given 0.03 cc. of a 10^{-2} dilution of virus (10^4 to 10^5 M.C.L.D.) intranasally.   2 days later, when it was expected that virus was present in the anterior rhinencephalon but not in the remainder of the brain, six of these mice and six normal 1 year old controls were injected intracerebrally with 0.03 cc. of 10^{-5} dilution of glycerolated virus (about 1 M.C.L.D.).   4 days after nasal instillation the remaining six mice, along with six normal control animals, were given a similar intracerebral injection.

As will be observed in Table VI, the amount of virus injected intracerebrally was in the minimal infective range, since only four of the six control animals succumbed in the first, and three of six control mice

TABLE VII

Effect of Intracerebral Injection of a Minute Dose of Vesicular Stomatitis Virus (Indiana Strain) in Old Mice Given Virus Intranasally 3 and 5 Days Previously

Age of mice	Virus given — Intranasally	Intracerebrally	No. of mice in group	No. of mice showing C.N.S. signs or dead on given date (November)														
				5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
15 days	10⁴ to 10⁹ M.C.L.D. on Nov. 4, 1936	None	6	0	0	0	6 S.*	6 d.†										
	10⁴ to 10⁹ M.C.L.D. on Nov. 4, 1936	None	10	0	0	0	0	0	0	1 S.		1 d. 1 S.					2 d.	
	10⁴ to 10⁹ M.C.L.D. on Nov. 4, 1936	1 to 10 M.C.L.D. on Nov. 7, 1936	5	0	0	0 ↑	0	0	2 d. 2 S.	3 d. 1 S.		4 d.						
Approximately 1 yr.	None	1 to 10 M.C.L.D. on Nov. 7, 1936	5			↑			4 d.		4 d. 1 S.	5 d.	2 d. 3 p.p.					
	10⁴ to 10⁹ M.C.L.D. on Nov. 4, 1936	1 to 10 M.C.L.D. on Nov. 9, 1936	5	0	0	0	0	↑	0	1 S.	3 S.	5 S. 3 p.p.‡	2 d. 3 p.p.					
	None	1 to 10 M.C.L.D. on Nov. 9, 1936	5					↑	0	0	3 d. 1 S.	4 d. 1 S.				5 d.		3 d. 2 p.p.

↑ = day on which intracerebral test dose was given.

* 6 S. indicates six animals with signs of encephalomyelitis.

† 6 d. indicates six mice dead.

‡ 3 p.p. indicates three mice showed paralysis of the posterior extremities.

in the second group.   Of six mice which had received virus intranasally 2 days prior to the intracerebral injection, four succumbed, or the same number as among the control animals, while of the six mice inoculated intracerebrally 4 days after the nasal dose, five succumbed, as compared with three of the six control animals.   These results suggest, therefore, that no appreciable tissue immunity existed in the brain at the time when the progression of virus appears to be held up.   One objection, however, is that the experiment as carried out gives no clue to the number of mice that might have died as a result of the nasal instillation *per se*.   Although on previous occasions a mortality of 75 per cent among nasally infected old mice never occurred, it was decided to repeat this experiment with an attempt to control this point.

Six 15 day old and twenty 1 year old mice were each given intranasally 0.03 cc. of 10^{-2} dilution (10^4 to 10^5 M.C.L.D.) of the Indiana strain.   The six young mice and ten of the old ones served as controls for the effect of the nasally instilled virus. At intervals of 3 and 5 days, five old mice, along with five 1 year old, normal controls, were injected intracerebrally with 0.05 cc. of 10^{-5} dilution of glycerolated virus (1 to 10 M.C.L.D.).

The results, summarized in Table VII, show that while all the young ones succumbed, only two of the ten 1 year old mice developed signs of encephalomyelitis.   In the intracerebrally injected group all of the ten normal control animals died and nine of the ten old mice, having had preliminary nasal instillation of virus, developed signs of encephalomyelitis.   It seems clearly evident, therefore, that there is no rapid development of tissue immunity in the brains of these old mice and that the factors concerned in the arrest of virus progression are probably preexistent and limited only to special areas of the brain.

DISCUSSION

The present investigation was undertaken primarily to discover in what way the pathogenesis of vesicular stomatitis virus disease in old, resistant mice varied from that in young, susceptible animals.   It was clear from the beginning that it was not a question of an acquired resistance of the entire C.N.S., since injections of virus directly into the brain found young and old animals equally susceptible.   It is only after the virus is given by a peripheral route that the resistance of the old animals becomes apparent.

The course of events after nasal instillation of the virus was investigated first. No evidence of a generalized systemic or blood infection could be found in either the young or old animals. 1 hour after nasal instillation of as much as 100,000 M.C.L.D. in young mice, no virus (or less than 60 to 70 M.C.L.D.) could be demonstrated in the nasal mucosa. Tests on the olfactory bulbs within a few minutes and 5 hours after instillation also failed to reveal any virus. 2 days later, however, it was abundant in both the nasal mucosa and the anterior rhineneephalon, but was either undemonstrable or present in only a trace in the remainder of the brain. On the 4th day, when the young mice showed definite clinical signs of C.N.S. involvement, virus was present in large amounts in the brain and nasal mucosa. In old mice the nasally instilled virus disappeared just as readily but failed later to become detectable in some of them while it reappeared in small amounts in others. It is significant, therefore, that virus was readily demonstrable in the anterior rhinencephalon of practically all the mice tested between the 2nd and 5th days. In the remainder of the brain, however, none was found during this period with one exception, in which only a minute amount was present as compared with the considerable quantity in the anterior rhinencephalic portion of the same brain. Other mice sacrificed on the 7th, 10th, and 16th days after nasal instillation showed no virus in any part of the brain. In one animal without any clinical signs of disease, when sacrificed on the 7th day, virus was found in both the anterior and posterior parts of the brain; this mouse might have been one of those which develop clinical signs of encephalitis as late as 10 or even 14 days after instillation. It was clear, however, particularly from experiments with smaller doses, that in old mice, which show no signs of disease after nasal instillation, the anterior rhinencephalon could contain considerable amounts of virus from the 2nd to the 5th day, which then could disappear without apparently involving the rest of the brain.

The question arose now as to why the virus present in the anterior rhinencephalon failed to infect the rest of the brain, especially since minimal amounts are capable of inducing a fatal encephalitis when injected directly into the brain. One explanation which suggested itself was that virus which reaches the brain from the nose may travel

in a closed system of nerve cells and cell processes, and in old mice may encounter zones in which its progression is inhibited, but when it is injected intracerebrally it may spread by way of the spinal fluid in and out of the ventricles and reach those parts of the C.N.S. which remain susceptible. Experiments on the fate of intracerebrally injected virus revealed that after such an inoculation it easily spread to remote parts of the C.N.S. It appeared, furthermore, that a certain amount of virus is either washed away (or rendered non-detectable), for when doses ranging from 10 to 1,000 M.C.L.D. were injected intracerebrally, none could be recovered by the methods employed 45 minutes later. Within 24 hours, however, virus became abundant and widely scattered throughout the brain. Yet after nasal instillation, readily demonstrable amounts of virus could remain in the anterior rhinencephalon without spreading to the rest of the brain. The suggestion was very strong, therefore, that under these circumstances virus was progressing in a closed system and was halted by some impenetrable barrier. The next question to be considered was whether this hypothetical barrier exists in the animal before it is exposed to the virus, or whether it represents a rapid acquisition of specific tissue immunity. In other words, does the virus fail to progress in old mice because the remainder of the brain has become immune by the time it is reached, or does it remain susceptible, the spread of the virus being inhibited by a preexisting, localized, insusceptible zone? In the experiments undertaken to answer this question, it was attempted to circumvent such a zone, if one existed, by direct intracerebral injection of minute amounts of virus in old mice at intervals of 2, 3, 4, and 5 days after nasal instillation, when the anterior rhinencephalon of these animals would be expected, from previous experience, to contain virus. If the brain had acquired an immunity in that short time, the intracerebral inoculation of this small amount of virus would be without effect and the mice should remain well. If, on the other hand, the brain as a whole were still susceptible, these animals would die, while the uninoculated, nasally instilled controls survive. The mice injected intracerebrally at the stated intervals after nasal instillation succumbed as did the uninstilled controls, and no evidence of a rapidly acquired cerebral immunity was therefore revealed. The

hypothesis of a preexisting barrier to the spread of the virus was thus strengthened.[2]

SUMMARY

1. After intracerebral injection or nasal instillation of vesicular stomatitis virus in young or old mice, there was no evidence of a generalized, systemic or blood infection.

2. Within 1 hour after nasal instillation of as much as 100,000 M.C.L.D. in young or old mice, no virus (i.e., less than 60 to 70 M.C.L.D.) could be demonstrated in the nasal mucosa. 2 days later and thereafter virus was abundant in the nasal mucosa of young mice, while among old mice it remained undemonstrable in some and present in small amount in others.

3. Virus was not detected in the anterior rhinencephalon of young and old mice within a few minutes and 5 hours after nasal instillation, but was almost uniformly demonstrable in this region, although not in the rest of the brain, on the 2nd day. This indicated that the primary invasion of the brain occurred by the olfactory rather than the fifth nerve pathway.

4. The essential difference in the further pathogenesis of the disease between the young mice which succumb with encephalomyelitis (5th day) and the old mice which survive without showing clinical signs of brain involvement, is in the progression of the virus from the anterior rhinencephalon. In the young the rest of the brain is invaded, while in the old resistant mice it is not, the progression of virus being arrested somewhere in the anterior rhinencephalon.

5. Since minimal amounts of virus injected intracerebrally were shown to be disseminated quickly through the entire brain, killing old as well as young mice, it was clear that virus so inoculated must spread differently from that which reaches the brain by the olfactory pathway.

[2] In discussing some of the present findings (previously reported in abstract form), Doerr (3) suggested that the development of a solid cerebral immunity rather than any hypothetical, preexisting barrier may be responsible for the arrested infection. The results of the present investigation revealed no evidence of such an immunity in the early stages.

6. That the arrest of virus progression in the brains of certain old mice is the result of a preexisting, localized barrier, developed with age, and is not due to a rapidly acquired, specific, cerebral immunity was shown by the failure of old mice to resist an intracerebral injection of 1 to 10 M.C.L.D., 2, 3, 4, or 5 days after preliminary nasal instillation.

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INFLUENCE OF HOST FACTORS ON NEUROINVASIVENESS OF VESICULAR STOMATITIS VIRUS

II. Effect of Age on the Invasion of the Peripheral and Central Nervous Systems by Virus Injected into the Leg Muscles or the Eye[*]

By ALBERT B. SABIN, M.D., and PETER K. OLITSKY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

The experiments reported in the preceding communication (1) indicated that after nasal instillation in mice the vesicular stomatitis viruses spread to the central nervous system (C.N.S.) by way of the first cranial nerve and subsequently along special pathways to other regions of the brain. The resistance of the greater number of old mice appeared to be due to an arrest of the progression of virus somewhere in the "anterior rhinencephalon." The present report deals with the mode of invasion of the C.N.S. of young mice and with the nature and site of the resistance exhibited by older animals after peripheral inoculation into other regions.

Methods

The animals and the general methods employed were described in the first paper (1). Only the following additional remarks are necessary.

To test for virus in the sciatic nerve, after inoculation into the calf muscles, it was bluntly dissected away from the lymph nodes and fat in the popliteal fossa, cut there and again cephalad near the vertebral column.

After intraocular injection, the brain was subdivided as indicated in the protocols, tables, and in Fig. 1. With the brain on its dorsum the pons, medulla, and cerebellum were removed, leaving *in situ* the corpora quadrigemina. Then

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turning the brain onto its ventral surface, the two cortical hemispheres, including Ammon's horn, were separated at the longitudinal fissure and peeled away from the mesencephalon and diencephalon.   Anteriorly the diencephalon was severed from the corpus striatum along the stria terminalis and at the level of the optic chiasm (Fig. 1).   When it was desired to test separately for virus the right and left halves of the mesencephalon and diencephalon, the cut was purposely not made in the midline in order to avoid, as best as could be done, admixture of the possible positive with the negative half.

EXPERIMENTAL

Spread of Virus after Intramuscular Injection in Young and Old Mice.—Old mice are more resistant to intramuscular injection than to nasal instillation of vesicular stomatitis virus.   Thus far not a single old mouse has been observed to show signs of disease or to succumb after intramuscular (also subcutaneous) injections of large amounts of virus.   To determine the nature and site of the resistance in intramuscularly injected old mice, it was decided to find out by what route virus deposited in the muscle reached the C.N.S. of very young mice and to investigate the variations which occurred in older animals. Clinical observation of young mice suggested that virus invaded the C.N.S. primarily by way of the nerves supplying the injected muscle, for after inoculation into the calf muscles of one posterior extremity the first sign is invariably flaccid paralysis of that leg followed by paralysis of the opposite member.   Signs of upper neurone involvement are always a late manifestation.

Young mice (15 days of age) and old mice (about 1 year old) were each injected into the muscles of the right posterior extremity with 0.3 cc. of a 10 per cent virus suspension (10^7 minimal cerebral lethal doses (M.C.L.D.)).   The young mice developed paralysis of the posterior extremities on the 4th day, while none of the old mice showed any signs of disease.   Young mice were killed 2 and 4 days, and old 2, 3, 4, 6, and 8 days after injection.   The material from two mice in each group was pooled for virus tests at the indicated intervals.   Each mouse was bled to death and the blood tested for virus by intracerebral injection of 0.03 cc. into each of three mice.   The other structures examined for virus included the inoculated calf muscles, the right popliteal lymph nodes (in the old), the right sciatic nerve, the lumbar spinal cord, and the left sciatic nerve.

The data presented in Table I indicate that intramuscularly injected virus has a different fate in young and old mice.   In the young, there was no evidence of virus multiplication in the blood and the spleens

contained virus on the 2nd, although not on the 4th day after inoculation; on the 2nd day virus was present in the right sciatic nerve, lumbar cord, and in a small amount also in the left sciatic nerve. On the 4th day, when the young mice exhibited flaccid paralysis of both posterior extremities, virus was easily demonstrable in the right sciatic
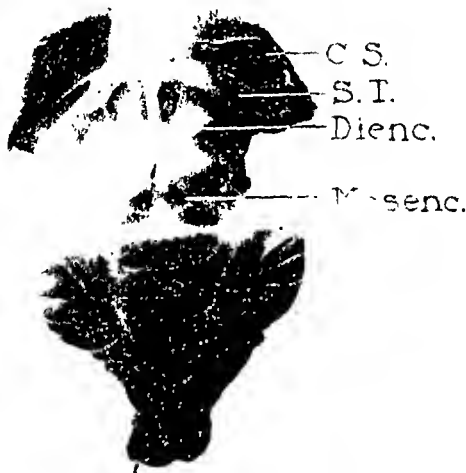
Fig. 1. Dorsal view of mouse brain with cerebral hemispheres removed. C. S., corpus striatum; S. T., stria terminalis; Dienc., diencephalon; Mesenc., mesencephalon.

nerve and spinal cord but none was found in the left sciatic nerves of the two mice studied. There is good evidence, therefore, that the primary invasion of the C.N.S. in the young was by way of the nerves supplying the inoculated muscles.

In the old animals, in spite of the injection of at least 10 million

TABLE I

Spread of Vesicular Stomatitis Virus (New Jersey Strain) in Young and Old Mice after Intramuscular Injection

0.3 cc. of a 10 per cent mouse brain suspension in broth, containing 10,000,000 M.C.L.D., was injected into the right leg muscles of each mouse

Tissue tested for virus	Time after inoculation	Age of mice supplying tissues	
		15 days	About 1 yr.
	days		
Heparinized blood	2	0, 0, 0	0, 0, 0
	3	n. t.	0, 0, 0
	4	0, 0, 0	0, 0, 0
	6	n. t.	0, 0, 0
	8	"	0, 0, 0
Right calf muscles (inoculated site)	2	2, 2, 2	2, 2, 2
	4	2, 2, 3	3, 5, 6
	8	n. t.	0, 0, 0
Right popliteal lymph nodes	2	"	2, 2, 2
	4	"	7, 0, 0
	8	"	0, 0, 0
Spleen	2	3, 4, 4	0, 0, 0
	4	0, 0, 0	0, 0, 0
	8	n. t.	0, 0, 0
Right sciatic nerve	2	2, 3, 3	0, 0, 0
	3	n. t.	0, 0, 0
	4	2, 2, 3	0, 0, 0
	6	n. t.	0, 0, 0
	8	"	0, 0, 0
Spinal cord	2	2, 2, 3	0, 0, 0
	3	n. t.	0, 0, 0
	4	2, 2, 2	0, 0, 0
	6	n. t.	0, 0, 0
	8	"	0, 0, 0
Left sciatic nerve	2	4, 4, 0	0, 0, 0
	3	n. t.	0, 0, 0
	4	0, 0, 0	0, 0, 0
	6	n. t.	0, 0, 0
	8	"	0. 0, 0

n. t. = no test was made.
Figures indicate the day of death of individual mice, 0 signifying survival.

cerebral lethal doses, virus was demonstrable only at the site of inoculation and in the regional popliteal lymph nodes, and not in the other tissues studied. The injected muscle contained it abundantly on the 2nd day, probably a little less on the 4th, while none was found on the 8th day. The regional lymph nodes showed virus on the 2nd day, only a trace on the 4th, and none on the 8th day. No virus was found at any time in the blood, spleen, lumbar cord, or sciatic nerves. Other tests with intramuscularly injected old mice, not recorded in the table, never revealed virus in the nervous tissue of these animals.

The resistance of old mice to intramuscularly injected vesicular stomatitis virus thus appears to be due to the inability of the virus to invade the nervous system. In this case, however, the barrier is peripheral, while after nasal instillation in old mice the virus can invade the peripheral nerve and reach the anterior rhinencephalon, where its further progression is arrested by a barrier in the central nervous system.

Effect of Intrasciatic Injection of Virus in Old Mice.—Little is as yet known of the mechanism whereby intramuscularly injected virus invades a peripheral nerve and of its subsequent transport to the C.N.S. To discover in what structures the progression of virus is held up, it appeared desirable to investigate the effect of direct intrasciatic inoculation.

The right sciatic nerve of 1 year old anesthetized mice was exposed just cephalad to the popliteal space and about 0.1 cc. of 10 per cent virus suspension (N. J. strain) was injected into it with a 28 gauge needle. The needle was moved up and down several times in order to cut some of the fibres. Although the injection was made with little pressure, the sheath of the nerve invariably broke and most of the material escaped. The edges of the wound were approximated and covered with collodion which proved to be adequate in keeping it closed and uncontaminated. The mice quickly recovered from the operation and showed no apparent immediate weakness of the inoculated extremity.

In the first experiment (Table II) five old mice were injected in the manner just described and three of them developed paralysis of the posterior extremities. One of these three died and the other two were sacrificed to test for virus in selected tissues. It was rather striking that in this group all mice exhibited evidence of biting either the tail or one or more of the extremities. The fact that two of the mice survived without showing any other signs of disease cast doubt on the possibility that the biting was a manifestation of virus involvement. This doubt was further increased when in subsequent experiments none of the mice exhibited

TABLE II

Effect of Intrasciatic Inoculation of Vesicular Stomatitis Virus (New Jersey Strain) in Old Mice

All mice injected into right sciatic nerve with 10 per cent virus suspension

Experiment	Mouse No.	Result	Presence of virus in					
			Left sciatic nerve	Right sciatic nerve	Lumbar cord	Cervical cord	Entire brain	Spleen
I	1	Paresis 4th day and bitten tail. Bitten right posterior extremity 5th day. Dead 6th day	n. t.	0, 0, 0	2, 2, 3	n. t.	n. t.	n. t.
	2	Paresis 4th day and bitten tail. Sacrificed 6th day	"	0, 0, 0	2, 2, 2	"	"	0, 0, 0
	3	Flaccid paralysis of right posterior (inoculated) extremity and biting of left anterior extremity 7th day. Sacrificed 7th day	0, 0, 0	0, 0, 0	2, 2, 2	2, 3, 4	2, 2, 3	0, 0, 0
	4	Bitten left anterior extremity 7th day. No paralysis. Survived						
	5	Chewed away left and bit right anterior extremity. No paralysis. Survived						
II	6	Flaccid paralysis of right posterior (inoculated) extremity 5th day. Paralysis both posterior and one anterior extremity 6th day. Complete paralysis 7th day. Dead 8th day.						
	7	Flaccid paralysis of inoculated extremity 6th day. Sacrificed 6th day	0, 0, 0	0, 0, 0	2, 2, 2	n. t.	n. t.	0, 0, 0
	8	Flaccid paralysis of inoculated extremity 7th day. Complete paralysis 8th day. Sacrificed 9th day	0, 0, 0	0, 0, 0	3, 3, 3	2, 2, 2	2, 2, 2	0, 0, 0
	9	Remained well. Sacrificed 9th day	n. t.	. n. t.	0, 0, 0	0, 0, 0	0, 0, 0	n. t.
	10	Remained well						
	11	" "						

Abbreviations as in preceding table.

these signs. In the second experiment three of six mice inoculated into the right sciatic nerve developed an ascending paralysis. In each of these three mice flaccid paralysis first appeared in the inoculated limb; in one on the 5th, in another on the 6th, and in the third mouse on the 7th day after inoculation.

The results of tests for virus in the sciatic nerves, spinal cord, brain, and spleen of many of these mice (Table II) leave little doubt that the C.N.S. was invaded in the animals which developed paralysis. No virus was found in the spleen of any of the animals nor in the C.N.S. of a mouse which remained well after intrasciatic inoculation. The failure to detect virus in the inoculated sciatic nerves at a time when the lumbar cords were positive and the animals were paralyzed was difficult to explain. Since each sciatic nerve was ground in 0.15 cc. of broth, of which 0.09 cc. was injected intracerebrally in mice (0.03 cc. into each of three animals), it does not seem possible that very much virus could be present in it without being detectable.

To appraise the significance of the results of intrasciatic virus inoculation, it is necessary to have a definite conception of what happens when material is injected into such a nerve. There is at present no general agreement regarding the fate of intraneurally injected solutions or colloidal suspensions. While it is generally believed that the perineural lymphatics or spaces of a nerve like the sciatic do not ... ... clear that a direct ... ... The observation of certain investigators that intraneurally injected dyes may reach the nervous axis at once or within a short time has been explained by the fact that when sufficient pressure is exercised during such inoculations, existing barriers may be ruptured (2). It is important, therefore, to consider the possibility that in the present experiments the virus may have been introduced directly into the subarachnoid space, and that the results which were obtained throw no light on the capacity of the nerve fibres themselves to transmit the virus. At the same time it should be remembered that by the very nature of the intraneural injection as practiced here, a certain number of nerve fibres are cut and their axis cylinders (the direct extensions of nerve cells situated in the spinal cord or ganglia) are exposed to the virus. However this may be, it is evident that the nervous system is infected in only about half the number of animals.

The fact that signs of C.N.S. disturbance appear first in the inoculated extremity and are delayed for 5, 6, or 7 days favors more the hypothesis that the virus first involved the nerve cells whose axis cylinders were exposed during the inoculation, rather than that it produced infection by way of the spinal fluid in the subarachnoid space, although additional evidence is desirable to confirm this interpretation.

Fate of Intrasciatically Injected Virus.—It has been shown in the preceding paper (1) that after direct intracerebral inoculation of even minute amounts of virus in old mice, multiplication occurs within 24 hours, when the virus is already more or less widespread in the C.N.S. It appeared, therefore, that if after intrasciatic inoculation (employing about 3 million M.C.L.D.) some of the virus rapidly reached the subarachnoid space, it should be easily demonstrable in various parts of the C.N.S. within about 24 to 48 hours.

To test this point, ten old mice were injected in the right sciatic nerve with 0.1 cc. of 10 per cent virus suspension (about 3 million M.C.L.D.). Most of the inoculum escaped into the surrounding thigh muscles but some remained in the nerve itself. During the first 3 days one mouse was sacrificed every 24 hours and the following structures were tested for virus: right sciatic nerve, left sciatic nerve, lumbar cord, cervical cord, entire brain, and, in one instance, the spleen. Of the remaining seven mice, three developed paralysis 5, 6, and 7 days after intrasciatic inoculation. The first sign in each case was flaccid paralysis of the inoculated extremity. One mouse was etherized on the 6th day, at a time when the right posterior extremity alone was paralyzed, and the structures enumerated above were tested for virus. Another mouse, which showed the first signs on the 7th day, was sacrificed on the 9th day, when it was completely paralyzed, and the same structures were examined for virus with the exception that the brain was subdivided into four parts, pons and medulla, cerebellum, and the right and left halves of the remainder, which were studied separately.

The results (Table III) show that during the first 3 days after sciatic inoculation no virus was demonstrable in the spinal cord or brain but was present in the inoculated sciatic nerve. When paralysis first appeared, however, no virus was found in either sciatic nerve; it was abundant in the lumbar cord and in the brain but was present in only a trace in the cervical cord. The relative scarcity of virus in the cervical cord, at a time when the lumbar cord below it and some part of the brain above it contained much virus, is considered significant because it corresponds to an observation made by Hurst in experimental poliomyelitis where, after intrasciatic injection, virus was demonstrable in the lumbar cord, the motor cortex and thalamus of the opposite side, although not in the cervical cord (3). In the mouse sacrificed 2 days after the onset of paralysis, every part of the brain and cord contained a good deal of virus, while the sciatic nerves had none.

TABLE III

Fate of Vesicular Stomatitis Virus (New Jersey Strain) after Injection into Sciatic Nerves of Old Mice

Time after inoculation into right sciatic nerve	Signs	Presence of virus in									
		Right sciatic nerve	Left sciatic nerve	Lumbar cord	Cervical cord	Medulla and pons	Cerebellum	Right cerebral hemisphere	Left cerebral hemisphere	Entire brain	Spleen
days											
1	None	3, 4, 5	0, 0, 0	0, 0, 0	0, 0, 0					0, 0, 0	n. t.
2	"	7, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0					0, 0, 0	0, 0, 0
3	"	4, 6, 8	0, 0, 0	0, 0, 0	0, 0, 0					0, 0, 0	n. t.
6	Paralysis of right posterior extremity 6th day. Sacrificed	0, 0, 0	0, 0, 0	2, 2, 2	7, 0, 0					2, 2, 2	0, 0, 0
9	Paralysis of right posterior extremity 7th day. Complete paralysis 8th day. Sacrificed	0, 0, 0	0, 0, 0	3, 3, 3	2, 2, 2	2, 2, 2	2, 2, 2	2, 2, 2	2, 2, 2		n. t.

Abbreviations as in preceding tables.

These observations do not coincide with what one would expect to find if the virus reached the subarachnoid space simultaneously with or soon after the intrasciatic inoculation. They are more in accord with the view that the spinal cord was invaded by way of the nerve fibres exposed to the virus. The long delay before virus became demonstrable in the spinal cord and its apparent disappearance from the nerve at or before the onset of paralysis may appear confusing at first glance, but these facts in themselves do not contradict this conception. In the light of these findings and their interpretation, it would appear that after intramuscular injection in old mice, the barrier to invasion of the C.N.S. is not so much in the nerve fibres themselves as at their endings. For by circumventing them, as by direct nerve inoculation, the same disease may be induced in the old as in young, susceptible mice, although somewhat more slowly.

Fate of Virus at the Site of Intramuscular Injection in Young and Old Mice.—The factors which determine the penetration of certain nerves by a neurotropic virus are not as yet understood. The relationship of local multiplication in either non-nervous tissue or the nerve endings to invasion of the nervous system has been investigated but little. The purpose of the following experiments was to disclose any observable difference in the fate of virus injected into the muscles of young, susceptible mice in which the nerves are invaded, as compared with that introduced into old, resistant mice in which the nerves are not invaded. The primary question concerned the multiplication of the virus at the site of inoculation in the two groups of animals.

In the first experiment a known amount of virus was injected into the right calf muscles of young and old mice, and 2 days later two from each group were sacrificed, the inoculated muscles removed and ground with sufficient broth to make 10 per cent suspensions. One part of these suspensions was titrated for virus and another was injected intramuscularly into groups of three young and three old mice, respectively. This process was repeated at 2 day intervals until a sufficient number of passages had been made to give an indication of what happened. The test with young animals was begun with three 15 day old mice, each of which was injected intramuscularly with 0.1 cc. of a 1:100 mouse brain suspension (about 3×10^4 to 3×10^5 M.C.L.D. of the N. J. strain). At each passage two mice were sacrificed and three new ones subinoculated with 0.1 cc. of the 10 per cent muscle suspension. The third mouse was retained to determine whether or not, and when paralysis might occur. Approximately 1 year old mice were used for the "old" group and the same procedure was followed, except that more virus was given

TABLE IV

Local Multiplication of Virus after Intramuscular Injection in Young and Old Mice

Age of mice in which virus was passed from muscle to muscle	Material injected intramuscularly	Virus in inoculated muscle 2 days later Dilution tested					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
15 days	Mouse brain suspension 3×10^4 to 3×10^5 M.C.L.D.	n.t.	2, 2, 4	n.t	2, 2, 2	3, 7, 0	n.t.
	First passage muscle suspension	"	n.t.	"	n.t.	n.t.	"
	Second passage muscle suspension	2, 3, 5	"	2, 2, 2	0, 0, 0	3, 8, 0	"
	Third passage muscle suspension	2, 2, 2	"	2, 2, 3	n.t.	3, 0, 0	"
	Fourth passage muscle suspension	2, 2, 2	"	2, 4, 7	"	0, 0, 0	"
	Fifth passage muscle suspension	2, 3, 4	"	2, 3, 7	"	4, 0, 0	0, 0
	Sixth passage muscle suspension	2, 2, 3	"	2, 3, 4	"	3, 3, 3	3, 3, 0
About 1 yr.	Mouse brain suspension 10^4 to 10^5 M.C.L.D.	2, 2	"	2, 5, 0	0, 0, 0	n.t	n.t.
	First passage muscle suspension	4, 4, 5	"	0, 0, 0	0, 0, 0	"	"
	Second passage muscle suspension	0, 0, 0	0, 0, 0	n.t.	n.t.	"	"
	Fourth passage muscle suspension	0, 0, 0	n.t.	"	"	"	"

Abbreviations as in preceding tables.

with the primary intramuscular inoculation, *i.e.*, 0.3 cc. of a 1:10 mouse brain suspension or 10^6 to 10^7 M.C.L.D.   At each passage 0.3 cc. instead of 0.1 cc. of the muscle suspension was subinoculated into new animals.

The results presented in Table IV show clearly that multiplication of the virus occurred at the site of inoculation in the young mice. Six muscle to muscle passages were carried out, with evidence of multiplication each time.   The muscle suspension of the sixth passage contained virus in a dilution of 10^{-6} at least, and the mice which at each passage were left as controls developed typical, ascending, flaccid paralysis.   On the contrary, there is no evidence that multiplication occurred in the old mice.   2 days after the primary injection of 10^5 to 10^7 M.C.L.D., virus was demonstrable in the inoculated muscles in a dilution of 10^{-3}.   It was present in the 10^{-1} (*i.e.*, 10 per cent suspension of the muscle) but not in the 10^{-3} dilution after the first passage, and none was found in the muscle of later passages.

Although it is thus clear that there is a definite difference in the fate of virus injected intramuscularly in young and old mice, one cannot state with equal assurance on the basis of this experiment that the virus does not multiply at this site in old mice.   More virus may, perhaps, be required to initiate multiplication in old than in young mice, and the total increase may be insufficient to permit successful muscle to muscle passage.   To investigate this point further, another experiment was planned.

One group of old mice was injected intramuscularly with about 10^6 M.C.L.D. and another with 10^4.   At intervals of 2, 24, 48, and 96 hours after injection mice from each group were sacrificed and the amount of virus present at the site of inoculation was estimated by intracerebral inoculation into other mice.

Among the mice injected with 10^4 M.C.L.D., virus was found at the site of inoculation at 2 hours but not later, while among those injected with 10^6 M.C.L.D. it persisted for the 4 days during which tests were performed (Table V).   Since, however, more virus was present at 2 hours than at any subsequent test period, one cannot say that any multiplication occurred.   The fact that there appears to have been slightly more virus in the mouse sacrificed at 48 hours than in the one at 24 hours may, perhaps, represent an example of individual variation.

To determine whether the virus would also fail to multiply in other non-nervous tissues of old mice, it was injected intracutaneously and subcutaneously into the pads of the posterior extremities.

Mice injected with about 300,000 M.C.L.D. were sacrificed at 2, 22, 72, and 120 hour intervals and the amount of virus in the pads estimated by intracerebral ~~~~ ~~~~~~ dilutions of highly centrifuged suspensions. (Horizontal ~~~~~~~~ ~~~ ~~~~~

did not produce bacterial meningitis and thus ~~~~~~~~~ tion.)

In this instance there was somewhat more virus at 22 than at 2 hours (Table VI). None (*i.e.*, less than 10^2 M.C.L.D.) was found in the mouse tested at 72 hours, while the 120 hour pad contained as much virus as the 2 hour one. The results show a certain amount of ex-

TABLE V

Fate of Virus (New Jersey Strain) at Site of Intramuscular Injection in Old Mice

Amount of virus injected intramuscularly	Dilution of muscle tested for virus	Time after inoculation			
		2 hrs.	24 hrs.	48 hrs.	96 hrs.
1,000,000 M.C.L.D.	10^{-1}	2, 5	2, 0	2, 2	3, 4
	10^{-2}	3, 0	8, 0	2, 0	0, 0
	10^{-3}	3, 5	0, 0	3, 0	3, 0
	10^{-4}	0, 0	0, 0	0, 0	n. t.
10,000 M.C.L.D.	10^{-1}	3, 0	0, 0	0, 0	0, 0
	10^{-2}	6, 0	0, 0	0, 0	0, 0
	10^{-3}	0, 0	0, 0	0, 0	0, 0
	10^{-4}	0, 0	0, 0	0, 0	n. t.

Abbreviations as in preceding tables.

pected variation as regards persistence of virus in individual mice, but do not suggest that any appreciable multiplication occurred.

Whether or not this inability of the virus to multiply at the site of subcutaneous or intramuscular inoculation in old mice can be held accountable for its failure to invade the local nerves is not clear. Without histological studies one cannot say in what structures at these sites the virus multiplies in the young and not in the old mice. It has already been shown that in young mice there is no evidence of a generalized systemic infection after intranasal or intracerebral injection, and that even after intramuscular inoculation of 10^7 M.C.L.D. virus is not regularly present in the spleen. While one might assume, therefore, that even in the young mouse the vesicular stomatitis vi-

ruses may, perhaps, be strictly neurotropic, the extensive multiplication occurring at the site of intramuscular injection would not be in accord with such a view unless this multiplication occurred entirely in nervous structures. In the old mice, however, there appears to be little evidence to indicate that the virus is not strictly neurotropic.

Intraocular Injection of Virus in Young and Old Mice.—In the preceding experiments the virus was injected into regions supplied by two essentially different types of peripheral nerves. After inoculation into the muscles of the leg the virus comes into relation with peripheral spinal nerves or with their specialized nerve endings, while after intranasal instillation there are, in addition to the fifth and sympathetic nerves, the olfactory neurones of the first order with their short, dendritic processes at the very surface of the olfactory mucosa. In

TABLE VI

Fate of Virus (New Jersey Strain) Injected into Pads of Old Mice

Pad ground up in 2.5 cc. of broth, and centrifuged supernatant liquid tested for virus	Time after injection of 300,000 M.C.L.D.			
	2 hrs.	22 hrs.	72 hrs.	120 hrs.
Undiluted	3, 6	3, 4	0, 0	3, 4
10^{-1}	0, 0	5, 6	0, 0	0, 0
10^{-2}	0, 0	0, 0	0, 0	0, 0

Figures as in Table I.

the former instance, the indication was that some local change in the old mice acted as a barrier against invasion of those peripheral spinal nerves by the virus; this peripheral barrier is probably almost always effective, since in our experience thus far not a single old mouse developed any C.N.S. signs after intramuscular injection. After nasal instillation the evidence indicated that the virus followed the olfactory rather than the fifth nerve pathway, and the barrier in old mice (which is less effective than after intramuscular injection) was somewhere in the C.N.S. rather than peripheral. The experiments dealing with the nature of this hypothetical central barrier suggested that the virus was spreading in a closed system along definite pathways and that somewhere in its course, local conditions prevented its further progression. The special anatomical conditions in the eye appeared

to supply a good opportunity for investigating further the nature of virus spread along certain nerves and the modifications which may develop with age.

As regards the direct nervous pathways after intraocular injection, there are four routes to be considered. First, infection of the ganglion cells in the retina with subsequent spread along the fibres of the optic nerve; second, along the fibres of the fifth nerve; third, along the fibres of the third nerve supplying the ciliary body and iris; and fourth, the sympathetic nerves. The optic nerve is not properly a peripheral nerve and the retina may be looked upon as an extracranial portion of the C.N.S. which can be exposed to the virus by an injection into the vitreous. In the case of the retina, one may thus have conditions simulating those in the C.N.S., while with the other nerves the situation may correspond more closely to that in the peripheral nerves already studied in the leg muscles and nose.

It is often stated, however, that from the eye substances may enter the pia-arachnoid space surrounding the optic nerve and thus spread to the base and rest of the brain by way of the spinal fluid (4). If that were to apply to vesicular stomatitis virus injected into the vitreous, one would expect no variation in effect from that obtained after ordinary intracerebral inoculation, *i.e.*, lethal infection in both young and old mice. The purpose of the first experiment, therefore, was to determine whether the effect of intraocular injection in young and old mice would simulate more closely that of direct intracerebral inoculation rather than of injection by other peripheral routes.

A suspension of N. J. virus was titrated simultaneously in 15 day and 1 year old mice by intraocular injection. The inoculations were made into the vitreous of one eye, giving about 0.01 to 0.02 cc.: the eye would bulge and a varying amount of the fluid would escape. Tenfold dilutions of the virus, ranging from 10^{-1} to 10^{-7}, were injected into groups of three young and three old mice. The 10^{-5}, 10^{-6}, and 10^{-7} dilutions were also injected intracerebrally into 4 week old mice in order to compare the relative infectivity of the virus by both routes.

The results (Table VII) indicate that the effect of intraocular injection of this virus simulates that obtained after other forms of peripheral, rather than that which follows direct intracerebral inoculation. While one of the three old mice which received the 10^{-1} dilution succumbed to virus encephalitis, all the other old animals remained well. The young mice, however, developed fatal virus encephalitis. The intraocular titre of the virus was 10^{-5}, as compared with the intracerebral end-point of 10^{-6}. It is well to recall, however, that the amount injected in the eye was considerably less than that given in the brain. Other tests not recorded here indicated that while 15 day old mice succumbed uniformly after intraocular injection, 21

day old animals already showed evidence of acquired resistance; the disease developed after a longer time in some, and failed to manifest itself in a varying number of others.

Spread of Intraocularly Injected Virus in the Central Nervous System of Young and Old Mice.—The purpose now was to identify the pathway to the brain taken by virus injected into the vitreous; to trace its subsequent course within the brain itself, and to find out in what way the results varied in young susceptible and old resistant mice. The

TABLE VII

Susceptibility of Young and Old Mice to Intraocular Injection of Vesicular Stomatitis Virus (New Jersey Strain)

Dilution of virus suspension	Intracerebral titre 4 wks. old mice Dose: 0.03 cc.	Intraocular titration in young and old mice Dose: 0.005 cc. to 0.01 cc.	
		15 days old Average weight, 9 gm.	About 1 yr. old Average weight, 35 gm.
10^{-1}	n. t.	3, 5, 5	0, 0, 6*
10^{-2}	"	4, 6, 8	0, 0, 0
10^{-3}	"	4, 5, 0	0, 0, 0
10^{-4}	"	0, 0, 0†	0, 0, 0
10^{-5}	3, 4, 4, 7	5, 5, 11	0, 0, 0
10^{-6}	7, 8, 0	0, 0, 0	0, 0, 0
10^{-7}	0, 0, 0	0, 0, 0	0, 0, 0

Figures as in Table I.

* Mouse sick on 6th day; sacrificed and virus demonstrated in brain by passage.

† The failure of these mice to show signs of disease, while all three which received the 10^{-5} dose succumbed, appears paradoxical but represents a phenomenon not infrequently encountered in virus titrations.

question may well be asked whether the resistance of the old animals was again due to some sort of barrier to virus progression, and then whether that barrier was in the eye itself or in some region of the brain. The fact that old mice, with few exceptions, show no signs of disease after intraocular injection in itself casts doubt on the possibility of the virus spreading to and within the brain by way of the cerebrospinal fluid.

To obtain an idea of the path of progression, different parts of the brain were examined for virus at varying intervals after intraocular injection. The choice of parts to be studied for virus content was necessarily governed by the known central

connections of the nerves supplying the eye. The most important and also most numerous structures exposed to the virus after inoculation into the vitreous are the ganglion cells of the retina. The axons of these cells form the optic nerve and the majority of them cross at the optic chiasm and terminate in the lateral, geniculate ganglion, the lateral nucleus of the thalamus, and the superior colliculus of the opposite side. The number of fibres which remain uncrossed, depending upon the extent of binocular vision, is, as in the rat, probably very small (5). If the virus were to travel in a closed system within these axons, one would expect to find it first in the diencephalon and mesencephalon of the side opposite to that of the inoculated eye, while if it traveled interstitially or spilled over readily from the axons into the interstitial tissues of the optic nerve, it should, upon reaching the chiasm, spread more or less equally to both sides of the diencephalon and mesencephalon. Furthermore, if it reached one side before the other, one might expect that the occipital cortex of the corresponding side (*i.e.* contralateral to the injected eye) would contain virus before that of the opposite side. In some experiments, the entire diencephalon and mesencephalon were studied for virus content, while in others they were divided into the right and left sides, the cut being made to one side of the midline in order to avoid contaminating the negative with the possibly positive portion. With the virus introduced into the right eye, the cut was made a little to the right of the midline because the left side would be expected to be positive and the right negative.

A difficulty in the interpretation of results obtained with material dissected in this manner arises when one considers the termination of that part of the third nerve which supplies the structures of the ciliary body and iris. After reaching and connecting with cells in the small ciliary ganglion, fibres of the third nerve in the mouse, as in other similar rodents, end in a single Edinger-Westphal nucleus which is situated in the midline just ventral to the aqueduct of Sylvius (5). The above dissection would always include this nucleus in the suspected side and one could not say whether the presence of virus in it was due to passage along the optic or third nerve path. From this nucleus, however, virus would not be expected to involve one occipital cortex before the other. For this reason it was considered particularly important to test separately the right and left occipital cortex.

The structures injured by the needle in the course of the injection, including those in the eye which might come in contact with the virus, are supplied by branches of the fifth nerve. These fibres pass through the ciliary and Gasserian ganglia and terminate in the pons and medulla. The Gasserian ganglion and the part of the fifth nerve lying at the base of the skull were removed together, and the structures from each side were tested separately. If the pons and medulla showed virus before other parts of the brain, it would point to the fifth nerve as an early route of progression. The sympathetic nerves and ganglia were not investigated in these experiments.

Still another pathway had to be considered. It has already been stated that during intraocular inoculation some of the virus escaped from the eye; such virus might conceivably reach the nose through the nasolachrymal duct and might

TABLE VIII

Spread of Vesicular Stomatitis Virus (New Jersey Strain) after Intraocular Injection in Young and Old Mice

Age of mice	Time after injection of virus into right eye	Mouse	Optic nerve and chiasm	Right dien-cephalon and mesen-cephalon	Left dien-cephalon and mesen-cephalon	Entire dien-cephalon and mesen-cephalon	Right occipital cortex	Left occipital cortex	Pons and medulla	Right 5th nerve and Gasserian ganglion	Left 5th nerve and Gasserian ganglion	Cerebellum	Olfactory bulbs
							Presence of virus in						
15 days	*hrs.* 20	1	0, 0	, 0	0, 0	—	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0
	42	2	0, 0	2, 5	2, 4	—	3, 0	2, 3	2, 2	4, 0	3, 0	2, 3	4, 0
	72	3	2, 0	2, 4	2, 4	—	2, 4	2, 3	2, 4	2, 2	2, 0	2, 2	2, 2
21 days	24	4	5, 0, 0	0, 0, 0	0, 0, 0	—	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		5	0, 0, 0	0, 0, 0	3, 5, 0	—	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	n. t.	n. t.	n. t.
	48	6	0, 0, 0	—	—	2, 2, 2	0, 0, 0	3, 3, 3	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		7	0, 0, 0	0, 0, 0	3, 0, 0	—	0, 0, 0	0, 0, 0	0, 0, 0	3, 6, 0	n. t.	n. t.	0, 0, 0
	96	8	0, 0	0, 0	0, 0	—	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0
		9	n. t.	2, 2	2, 2	—	4, 6, 0	3, 3, 3	n. t.	n. t.	n. t.	n. t.	n. t.
About 1 yr.	*days* 2	10	2, 2, 2	—	—	3, 3, 4	5, 0, 0	3, 3, 4	4, 9, 0	3, 3, 4	7, 0, 0	3, 0	n. t.
		11	0, 0	0, 0	0, 0	—	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0
		12	0, 0	0, 0	0, 0	—	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0
	4	13	0, 0, 0	—	—	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	n. t.
	6	14	0, 0, 0	—	—	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	n. t.	n. t.	0, 0, 0	''

Abbreviations as in preceding tables.

invade the brain through the highly susceptible olfactory mucosa. In such an event one would expect the olfactory bulbs to show virus at an early stage.

With these anatomical considerations in mind, a number of experiments were performed, in which the parts of the brain just enumerated, and occasionally other parts as well, were tested for virus at different intervals after intraocular injection. The inoculations were always made into the right eye with a 10 per cent suspension of the virus, so that the amount administered varied roughly from 10^5 to 10^6 M.C.L.D. 15 day, 21 day, and 1 year old mice were used, and the results are summarized in Table VIII.

The 15 day old mice were sacrificed at 20, 42, and 72 hours after inoculation. Control mice all had pronounced C.N.S. signs at 72 hours. At 20 hours no virus was demonstrable in any of the structures examined. At 42 and 72 hours the virus was so diffusely scattered that one could not be certain about the paths which might have been primarily traversed. It has already been remarked that in 21 day old mice the disease has a slower evolution and occasionally even fails to develop. It was thought, therefore, that this might be associated with a slower progression of virus within the brain and might define more easily the routes taken. That this proved to be the case is evident from the results recorded in Table VIII. In one 21 day old mouse sacrificed at 24 hours, a trace of virus might have been present in the optic nerve and chiasm, while none was demonstrable in any of the other structures studied. In another mouse etherized at 24 hours it was demonstrable only in the left diencephalon and mesencephalon but not in the same structures of the right side or in any of the other parts tested. At 48 hours one mouse showed virus in the diencephalon and mesencephalon (tested *in toto*) and in the left, but not in the right occipital cortex, or in any of the other structures; another showed a trace of virus only in the left side of the diencephalon and mesencephalon and in the right Gasserian ganglion and fifth nerve. At 96 hours one mouse (which might have proved resistant) exhibited no virus in any of the structures, while in another, in which only the right and left sides of the diencephalon and mesencephalon and the right and left occipital cortex were studied, it was demonstrable in all, but definitely less in amount in the right (homolateral) than in the left (contralateral) occipital cortex.

Integration of the results obtained with the 21 day old mice leads to the belief that the decussating optic pathway is primarily used.

In view of what has already been said of the position of the Edinger-Westphal nucleus in the mesencephalon, the implication of the optic path is based on finding virus only in the contralateral occipital cortex at an early stage. The uniform absence of virus from the olfactory bulbs in the early stages shows that there was no contamination from that source. It cannot be ascertained from these findings whether or not the third nerve pathway was traversed and the involvement of the fifth nerve was either dubious or only suggestive. It is not certain that a similar course is necessarily followed by the virus in the 15 day old mice, although such a possibility is not excluded by the available facts. It is clear, however, that in order to establish the pathways followed by the virus from the eye, the present findings should be supplemented by suitable pathological studies. These will form the subject of the next paper.

Among five 1 year old mice, the brains of which were examined on the 2nd, 4th, and 6th days, virus was found in only one, sacrificed on the 2nd day after intraocular injection. This may have been one of the exceptional mice which would have succumbed to the experimental disease. It should be noted again that while the contralateral occipital cortex of this animal had considerable virus, only a minute amount was present in the homolateral side. The absence of virus in the brain of two other mice sacrificed on the 2nd day, as well as in that of the other animals studied, suggests that after intraocular injection the barrier to cerebral invasion is probably in the eye of the old, resistant animals.

<div align="center">DISCUSSION</div>

After inoculation into the leg muscles of young mice, virus was found to undergo considerable multiplication at the site of injection and to invade within 2 days the spinal cord by way of the sciatic nerve, giving rise on the 4th day to flaccid paralysis of the inoculated extremity as the first clinical sign of the disease. The virus spread to the rest of the brain and the animals died. No virus was found in the blood, although the spleen was occasionally positive after injection of 10 million M.C.L.D. Old mice injected similarly never showed signs of disease, regardless of the dose administered. In them no definite evidence of local multiplication could be obtained and in spite of the injection of as much as 10 million M.C.L.D. and the local persistence

for days of many thousands of M.C.L.D. of virus, it invaded neither the sciatic nerve nor the C.N.S., and the animals remained well. The fact that half the number of mice developed the typical paralytic disease after direct intrasciatic inoculation (with trauma to the nerve) suggested that some barrier at the site of intramuscular injection in the old mice prevented the virus from invading the nerve. This assumption was strengthened by the finding that the intraneural injection was not followed by a direct and immediate spread of virus into the C.N.S.; the virus appeared to be localized in the nerve for at least 3 days, during which period none was found in the spinal cord. In animals which failed to develop the disease after intraneural injection, no virus was demonstrated in the C.N.S.

To identify the nature and precise location of the barrier at the site of intramuscular injection, one must have a clearer and better founded conception than is now available of the mechanism by which a peripheral nerve, like the sciatic, is invaded by virus deposited in the muscle. If the virus could progress to the C.N.S. only along the axons, it might first have to penetrate certain specialized nerve endings, which perhaps may not be accessible except through the cells that they supply. If this were the case, one could understand how the mere presence of a large amount of a virus without the simultaneous capacity to attack the cells might fail to involve the nerve endings. It should be mentioned, however, that rabies virus has been reported as being capable of invading a peripheral nerve without primary local multiplication (2). The absence of local multiplication in old mice cannot, therefore, in itself be considered as the underlying factor of the failure of large amounts of virus which persist in the muscle to invade the nerve. Furthermore, it will be shown in a subsequent publication that this same virus in another host, whose central nervous system as a whole is as susceptible as that of the mouse, is capable of local multiplication in peripheral tissue without at the same time invading the nerve. The rôle of the nerve endings, therefore, deserves especial consideration in further work on this subject.

The investigations on the intranasal and intramuscular routes of inoculation revealed two apparently distinct mechanisms of resistance in old mice. In the former instance, the virus can involve the C.N.S. but the animal remains well because its progression is halted in a

"silent" area, probably somewhere in the anterior rhinencephalon, while after intramuscular injection, the barrier is peripheral, the virus being unable to invade either the peripheral nerves or the C.N.S. The effect of intraocular injection was studied in young and old mice because the special anatomical structure and relations of the eye, which were discussed in the text, afforded an opportunity for further elucidation of the nature of virus progression and of the changes which the age of the host may induce. Injections of the virus into the vitreous of young mice proved almost as effective in producing virus encephalitis as direct intracerebral inoculation. Old mice, however, with few exceptions, proved to be resistant, even when large amounts of virus were given. Experiments dealing with the spread of the virus from the eye to the brain indicated that in young mice the primary pathway is probably along the axons of the optic nerve with decussation to the contralateral diencephalon and mesencephalon and subsequent early spread to the corresponding occipital cortex. In resistant, old mice, however, the virus meets some sort of barrier within the eye and fails to invade the brain.

The phenomena encountered in the present studies on the mode of progression of vesicular stomatitis virus after different routes of inoculation are difficult to explain except on the basis of the axonal spread of this virus, not only from the periphery to the brain, but also along specific closed or insulated tracts across synapses within the brain itself. The hypothesis of the development with age of certain localized barriers in the peripheral and central nervous systems as an explanation for the resistance of old mice to peripheral inoculations with this virus is tenable only in relation to such a concept. For this reason it was deemed advisable to attempt to obtain, if possible, pathological confirmation of the mechanisms which the purely biological experimentation suggested. The results of such a study are to be presented in a subsequent paper.

SUMMARY

1. Injection of vesicular stomatitis virus into the leg muscles of young mice gives rise to flaccid paralysis of the inoculated extremity as the first clinical sign of a disease which is invariably fatal; old mice similarly injected with the largest doses of virus survive without exhibiting any signs of illness.

2. In young mice the virus was shown to multiply at the site of inoculation and to invade the sciatic nerve and spinal cord; there was no evidence of multiplication of virus in the blood or viscera.

3. In old mice, after intramuscular injection of as much as 10 million M.C.L.D., there was no evidence of either local or systemic multiplication; in spite of the persistence of thousands of M.C.L.D. of virus at the site of inoculation for at least 4 days, there was no detectable invasion of the sciatic nerve or the central nervous system.

4. Injection of the virus directly into the sciatic nerve of old mice led to the typical paralytic disease in half the number of animals.

5. For 3 days after intrasciatic injection the virus could be demonstrated in the nerve but not in the spinal cord or brain.   At the onset of paralysis (6th day) virus was detectable in the spinal cord but no longer in the inoculated nerve.

6. The capacity of the virus to invade the central nervous system from the nerve but not from the muscle suggested the existence of a barrier in the muscle or myoneural junction.

7. Injection of the virus into the vitreous humor of the eye is followed by a fatal encephalitis in 15 day old mice, but 1 year old mice, with few exceptions, survive without showing signs of disease.

8. The spread of virus in the brains of intraocularly injected, 15 day old mice was too rapid to indicate the pathways which were pursued, but in 21 day old mice there was evidence that the primary pathway was probably along the axons of the optic nerve with decussation to the contralateral diencephalon and mesencephalon, and subsequent early spread to the corresponding occipital cortex.   In resistant, old mice, however, no virus was found in any part of the brain.

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PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

XIII. Sedimentation of the Tumor Agent, and Separation from the Associated Inhibitor[*]

By ALBERT CLAUDE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

Isolation of the agent causing Chicken Sarcoma I has been attempted repeatedly in this laboratory. By the use of methods of adsorption, gelatin precipitation, and dialysis, the tumor principle was successfully separated from a number of inactive or inhibitory materials (1). A study of the absorption power of the tumor extracts for ultraviolet light failed to establish a definite correlation between the absorption spectrum and the tumor-producing activity of the solutions, purified fractions of comparatively high tumor-producing power being practically transparent to ultraviolet light (2). These results suggested that the actual amount of the principle in the solutions tested was probably very small. Recently Ledingham and Gye have applied a method of high speed centrifugation to the separation of the agents causing the Rous (Chicken Tumor I) and Fujinami tumors (3). Furthermore, McIntosh has obtained evidence of the concentration of the agents of several transmissible chicken tumors in the air-driven centrifuge of Henriot and Huguenard (4).

Since centrifugation seemed to afford a convenient means for the concentration as well as purification of the chicken tumor agent, the conditions of its sedimentation in intense centrifugal field have been investigated. The results presented in this paper show that separation of the active principle, present in ordinary tumor extract, can be accomplished in a relatively short time by the use of a centrifugal

force not exceeding 15,000 times that of gravity. Under these conditions the inhibiting principle, usually associated in the tumor extract with the active agent, fails to be concentrated in appreciable proportion. The difference in the sedimentation constants of the tumor agent and its inhibitor was indicated by the greater tumor-producing power of the isolated sediment as compared with that of the unspun preparation.

Material and Methods

Tumor Extracts.—Fast growing tumors were used for these tests. The tumors were produced by inoculating 1.5 cc. of fresh tumor pulp in each side of the breast muscle of an adult Plymouth Rock hen. Normally the tumors so produced developed rapidly, the bird rarely surviving the injection more than 12 to 13 days. Well developed tumors were obtained between the 7th and the 12th day following inoculation. As a rule the tumors proved to be bacteriologically sterile, as shown by negative tests on agar and in broth. The fresh tumor tissue, or the tissue kept frozen until used (no more than 24 hours), was extracted with a volume of distilled water equivalent to 15 times the weight of the pulp. No alkali was added. The extract was centrifuged 20 minutes at 3,500 R.P.M. and passed through sterile gauze. The extract was filtered through a Berkefeld V candle, under negative pressure, the suction flask being kept on ice. The characters of the filtrates differed somewhat, according to the particular tumor used and to the porosity of the Berkefeld filter. The total solids of the filtrates varied from 1.3 to 2.7 mg. per cc., with an average dry weight of 2.1 mg. per cc. The viscosity of the Berkefeld filtrate, which varied greatly according to the amount of mucin present, was found to be from 1.12 to 3.04 times that of water at 20°C. as determined in the viscosimeter of Ostwald. In certain cases the time of centrifugation at high speed was adapted to meet the particular high viscosity of the fluid.

Centrifuge.—The device used for this work was the multispeed attachment and No. 295 head provided by the International Equipment Company, Boston, Massachusetts, for their type B, size 1 centrifuge. The whole centrifuge was installed in a cold room, the temperature of which changed slowly from 5–9°C. during the day when the centrifuge was in use. Under these conditions the temperature of the revolving head and of the fluid inside soon reached an equilibrium near 20°C., which was approximately maintained even when the centrifugation was continued without interruption for 4 hours. The average speed of the top, under cold room conditions, was 17,000 R.P.M. The glass tube occupied an inclined position at 51° angle with the axis of rotation. The inside diameter of the tubes used was approximately 1.2 cm., so that during centrifugation the maximum height of the column of liquid, perpendicular to the axis of rotation, was 1.9 cm. At 17,000 R.P.M. the centrifugal force applied at the center of the tube was about

14,000 times the pull of gravity. In later experiments cellulose containers were used in place of the glass tubes and rubber caps, thus increasing the capacity of the centrifuge.

Activity Tests.—Tests for tumor-producing powers were made by injecting 0.4 cc. of the test solution intracutaneously in adult Plymouth Rock hens. Each bird was injected in six areas in the breast region. This always included two areas injected with the untreated Berkefeld filtrate, as control. In recording the results, an arbitrary index of activity was obtained by combining the number of positive inoculations and the average surface of the tumors.

TABLE I

Displacement and Concentration of the Chicken Tumor Agent by High Speed Centrifugation

Preparation tested	Character of test preparation	Inoculation tests (16 to 32 day tumors)			
		No. of areas injected	No. of tumors	Positive inoculations	Average size of tumors
				per cent	*cm.*
Berkefeld filtrate............	Transparent; Tyndall effect positive	24	24	100	1.6 x 1.4
Surface layer..............	Clear; no Tyndall effect	18	4	22	0.9 x 0.8
Concentrated sediment.......	Opalescent	24	24	100	2 3 x 1.6

RESULTS

Preliminary tests indicated a displacement of the tumor agent in filtrates submitted to a high centrifugal force and its concentration at the bottom of the centrifuge tube.

The results recorded in Table I are computed from four experiments in which an active Berkefeld filtrate was spun at 17,000 R.P.M. for 2½ or 3½ hours. At the end of the centrifugation period 0.5 cc. of fluid, representing the upper part of the tube, was removed by means of a syringe, provided with a No. 26 gauge needle, and combined with the corresponding portions from the other tubes. The next 4.5 cc., or the remaining 5.0 cc. of the filtrate, was removed by the same technique and discarded. The sediment was resuspended in 0.5 cc. of the filtrate, left at the bottom of the tube for that purpose, or when the whole of the filtrate was discarded, in Tyrode's solution of pH 8.8. The product was combined, as was the surface fluid, and the suspension used for inoculation tests. The final volume of the Tyrode's solution in which the sediment was contained repre-

sented, according to the experiment, 1/4 or 1/10 the volume of the original filtrate. At the end of the centrifugation the temperature of the filtrate in the centrifuge was found to lie between 19° and 22°C., depending on the temperature of the cold room at the start. During the experiment a sample of filtrate, set aside as control, was left in the dark in a water bath regulated to a temperature of 20°C.

As shown in Table I, the effect of centrifugation was to decrease considerably the tumor-producing power of the filtrate at the top, and concentrate the tumor agent in the bottom fluid or in the sediment. The distribution of the tumor agent in the filtrate was next studied by testing the tumor-producing activity of successive sections of the column of fluid after centrifugation. The protocol of a typical test will be given, because it illustrates the trend of results which were obtained in subsequent experiments.

The procedure adopted in the experiment is given in Table II. The column of filtrate submitted to 3 hours centrifugation was fractionated by removing from the tubes successive layers of fluid by means of a syringe provided with a fine needle (No. 26 gauge). The corresponding portions from each tube were combined and used for activity tests. The sediment was suspended in Tyrode's solution and the coarse particles were discarded by 5 minutes centrifugation at 3,000 R.P.M. The substance was deposited again by $2\frac{1}{2}$ hours centrifugation at 17,000 R.P.M., and the wash solution removed by decantation. Before taking up the sediment, the tubes were always inverted to drain off the last drop of fluid, and their inside walls wiped with sterile gauze. The washed sediment from 33 cc. filtrate was resuspended in 5 cc. Tyrode's solution. In this concentrated form the preparation was homogeneous, transparent, and slightly opalescent when seen against a dark background. An attempt to determine the absorption spectrum of the solution showed that the "concentrated sediment" had no more absorbing power for ultraviolet light than the Tyrode solution used as control.[1]

Before injection, 5.6 cc. Tyrode's solution were added to 1 cc. of the concentrated preparation to obtain a dilution of the sediment comparable to that existing in the original filtrate. In this diluted solution the dry weight of the material isolated from the filtrate by high speed centrifugation was 0.0008 mg. per cc.

The activity of each fraction tested is included in Table II. Six additional experiments were performed by following exactly the procedure outlined in Table II, but adjusting the time of efficient

[1] The ultraviolet absorption tests were made by Dr. A. Rothen.

centrifugation to 2, 3, or 4 hours. In every case, notwithstanding the increase in the time of centrifugation, the results were practically duplicates of those given in Table II. From the analysis of the results, one may assume that, after centrifugation, at least 10 per cent of the apparent tumor-producing activity was left in the filtrate. A definite concentration of the tumor principle had occurred in the bottom of the tube, as shown by the increased activity of the sixth layer of fluid. In spite of the successive manipulations to which the sediment was submitted, and loss of some activity in the process, the washed sediment exhibited a tumor-producing power greater than that of the original filtrate. This enhanced activity of the sediment was confirmed and emphasized in the following experiments.

Table III gives the combined results of five experiments. The procedure followed was similar to that already given in the text and outlined in Table II. According to the experiment concerned, minor variations were introduced in the time of centrifugation, the number and length of preliminary centrifugation, and the number of washings of the sediment. In two cases the preliminary centrifugation at 17,000 r.p.m. was continued for 20 and 25 minutes, respectively, and the experiment carried out with this more homogeneous filtrate. The time of effective centrifugation varied from $2\frac{1}{2}$ to 4 hours. The deposited material was taken up in 5 or 10 cc. of Tyrode's solution and the coarser particles discarded by 5 to 10 minutes centrifugation at 3,000 r.p.m. The clarified sediment in Tyrode's was again separated by $2\frac{1}{2}$ to 4 hours centrifugation at 17,000 r.p.m. This washed sediment was resuspended in a volume of Tyrode's equal to that of the original filtrate and tested for tumor-producing activity. The Berkefeld filtrates used, after removal of the first sediment, were either clear or slightly opalescent. The washed sediment always gave an opalescent solution.

The results summarized in Table III indicate that the chicken tumor agent is more effective when separated from the other constituents of the tumor extract. The increase in tumor-producing activity was not always striking, but it was constant throughout these experiments and was apparent in the size of the tumors and the percentages of takes. The results are more significant when one takes into account the fact that, even after 4 hours of centrifugation, the supernatant fluid was not wholly deprived of tumor-producing activity. The difference in tumor-producing power of the untreated filtrate and the washed sediment did not disappear on progressive dilution of these materials. On one occasion, both fractions from an experiment,

TABLE II

Fractionation of Chicken Tumor Filtrate by High Speed Centrifugation (Experiment B 7)

Fraction No.	Inoculation tests (24 day old tumors)			
	No. of areas injected	Positive inoculations	Average size of tumors	Index of activity
		per cent	cm.	
1	10	100	1.8 x 1.5	270
2	4	25	1.2 x 0.8	24
3	4	50	1.4 x 1.2	84
4	4	50	0.8 x 0.7	28
5	4	50	1.4 x 1.1	77
			No test	
6	4	100	2.3 x 1.7	391

33 cc. Berkefeld filtrate { clear; pH 7.3; total solids, 2.30 mg. per cc.; viscosity = 1.87

5 min. 17,000 R.P.M.

Sediment { minute deposit light brown (discarded

Supernatant fluid { very clear; Tyndall effect uncertain

3 hrs. 17,000 R.P.M.

Surface layer (1 cc.)
2nd " (1 ")
3rd " (1 ")
4th " (1 ")
5th " (1 ")
6th " (0.5 cc.)

5.5 cc. tube

Sediment { thin film; white; 2 mm. in diameter

7	2	100	0.9 x 0 8	72
8	6	100	2.0 x 1.6	320
9	4	25	1.1 x 0.9	25

Sediment from 33 cc. filtrate (combined in 5.5 cc. Tyrode's solution of pH 8.8)

5 min. 3,000 R.P.M.

Supernatant fluid {homogeneous; Tyndall effect positive}

Sediment (discarded)

2½ hrs. 17,000 R.P.M.

Wash water (clear)

Washed sediment {resuspended in 5.5 cc. Tyrode's solution; homogeneous; transparent; Tyndall effect positive; no absorbing power for ultraviolet light}

Washed sediment, diluted {volume made up to 33 cc. with Tyrode's solution; total solids 0.0008 mg. per cc.}

Berkfeld filtrate, diluted {1:10 with Tyrode's solution; total solids 0.23 mg. per cc.}

included in Table III, were tested at 1:10, 1:20, and 1:50 dilutions. The size of the tumor decreased in both series, but the greater potency of the washed sediment remained manifest. The effect of washing on the tumor-producing power of the sediment was illustrated by the results of an additional test, in which the sediment, clarified by centrifugation at low speed, was tested before and after the last high speed centrifugation. The average size of the tumors produced after 17 days by the clarified sediment and by the sediment after washing was 2.2 x 1.7 and 2.8 x 2.1 cm., respectively.

The conditions of the separation of the tumor agent in the centrifuge have been controlled by the use of the respiratory pigments of the snail and of the horseshoe crab, proteins of high molecular weight

TABLE III

Separation of the Tumor Agent in More Effective Form by Centrifugation and Washing of the Sediment

Test solutions	Character of preparations	Inoculation tests (17 to 30 day old tumors)			
		No. of injections	Positive inoculation	Average size of tumors	Index of activity
			per cent	cm.	
Berkefeld filtrate.......	Clear to slightly opalescent	38	89	2.1 x 1.6	299
Surface layer...........	Clear	24	30	1.0 x 0.8	24
Washed sediment........	Opalescent	46	98	2.6 x 1 9	484

which, in the oxidized form, have a deep blue color. An analysis of the results obtained with these substances follows.

Sedimentation of Hemocyanins.—When the hemocyanins of *Helix* and of *Limulus* were submitted to high speed centrifugation, it was found that the pigments would separate with a sharp boundary which remained unblurred for some time after the centrifuge was stopped.

The snail hemocyanins were obtained from the blood of the three species, *Helix pomatia, aspersa,* and *lactea.* The serum containing the respiratory pigment was cleared by centrifugation and used without further dilution. The viscosity of such preparations was found to vary from 1.12 to 1.2, and the dry weight varied from 19 to 20.6 mg. per cc. Sedimentation tests were made with the reaction of the solution adjusted to pH 8.4, 6.6, and 5.3. *Limulus polyphemus* blood was withdrawn directly from the heart. The coagulum was discarded by

centrifugation and the serum stored at 0°C. under toluol. Dialyzed *Limulus* serum had a dry weight of 85 to 99 mg. per cc., and a viscosity of 1.2 to 1.4. The sedimentation tests were made with the dialyzed sera in concentrated form or at 1/2 or 1/10 dilutions, or with a 0.98 per cent solution of the recrystallized protein, and carried out with the reaction of the solution adjusted to pH 7.4 or 6.6.

The hemocyanin solutions were submitted to centrifugation at 17,000 r.p.m. for periods of 1, 2, 3, and 4 hours. The speed of centrifugation of the hemocyanin molecules was determined by direct measurement of the distance between the boundary of sedimentation and the meniscus. In each case the volume of clear solution above the boundary could be withdrawn without mixing the solution, as shown by the fact that the sedimenting boundary remained unblurred and that the sample obtained was pigment-free. The procedure used in obtaining samples of tumor filtrates from the centrifuge for inoculation tests was followed

TABLE IV

Speed of Sedimentation of Limulus and Helix Hemocyanins

Time of centrifugation (278 R.P.sec.)	*Limulus* hemocyanin (molecular weight = 2.04 × 10⁶)			*Helix* hemocyanin (molecular weight = 5 × 10⁶)	
	Distance between surface of fluid and boundary		Volume of pigment-free solution withdrawn	Distance between surface of fluid and boundary	Volume of pigment-free solution withdrawn
	Calculated	Found			
hrs.	*cm.*	*cm.*	*cc.*	*cm.*	*cc.*
1	0.18	0.13	0.16	0.40	0.45
2	0.39	0.37	0.45	0.67	0.80
3	0.59	0.60	0.67	1.30	1.60
4	0.79	0.80	0.90	Completely sedimented	

closely in removing the clear zone. As a rule, a sedimentation run of tumor filtrate comprised also a tube of hemocyanin solution as a control. The results of the measurements are summarized in Table IV.

According to Svedberg and Chirnoaga (5) the respiratory pigment of *Helix pomatia*, near the isoelectric point, has a molecular weight of 5 × 10⁶ and a diameter of 24 mμ. The principal component of *Limulus* serum has a molecular weight of 2.04 × 10⁶, as determined by Svedberg and Heyroth (6). The diameter of the *Limulus* hemocyanin molecule, computed from the results of Svedberg, was estimated at 16.8 mμ. According to Stoke's formula, the velocity of *Helix* and *Limulus* hemocyanins in our centrifuge (278 R.P.sec.; viscosity 0.01; density of particle 1.36; mean distance from axis of rotation 4.4 cm.) would be 1.4×10^{-4} and 7.7×10^{-5} cm. per second, respectively, and the

minimum time for their complete sedimentation would be 3 hours and 45 minutes and 6 hours and 50 minutes. The findings presented in Table IV are in fair agreement with these figures. *Helix* hemocyanin was completely sedimented by 4 hours centrifugation and *Limulus* hemocyanin solutions required 7 hours for their complete separation. These results indicate that the centrifugation method used in this work is an adequate means for the separation of protein particles of 24 mμ or more in diameter. The fact that the hemocyanins separated with a sharp boundary indicates that convection currents, due to evaporation or heat gradients, and the vibrations communicated by the centrifuge during operation or while stopping, were negligible. Rotation communicated to the column of liquid during deceleration had no appreciable effect on the equilibrium reached in the fluid at the moment of the stop. The particularly sharp boundary presented by *Limulus* hemocyanin solutions indicated the homogeneity of the pigment under observation. This boundary remained unblurred as long as 8 minutes after stopping the centrifuge, when diffusion became noticeable. The same sharpness of boundary was not observed in the sedimentation of *Helix* hemocyanin, blurring being especially pronounced at the late stages of centrifugation. This may have been caused by the presence of molecules of smaller size in the original material or by the formation, during centrifugation, of dissociation products, such as are known to occur under the effect of dilution.

Under the same conditions no clear cut separation of the tumor agent was obtained. When tumor filtrates were centrifuged under conditions which proved to be sufficient to effect the separation of *Helix* hemocyanin, some tumor-producing activity was found to persist in the solution, even after a 4 hour run, although about 90 per cent of the tumor agent had been deposited during the 1st hour. This may indicate that an unrecognized factor, which does not affect the sedimentation of hemocyanin, is effective in preventing the complete separation of the tumor agent. It is also possible that part of the tumor agent is present in the solution in the form of units of smaller size, which are not appreciably affected by a centrifugal force sufficient to throw down particles of the size of *Helix* hemocyanin molecules.

Physical Characters of the Active Fraction of the Chicken Tumor Material.—An agent large enough to be affected by a centrifugal field, such as that applied in this work, may be expected to have a pronounced scattering effect on light when in solution. Observation of the Berkefeld filtrates and of the products of sedimentation seems to indicate that the tumor-producing activity is associated, to a certain extent, with the opalescence of the solution. The solutions were examined against dark background without special illuminating device, or else with a lamp provided with a condenser. As a whole, active filtrates were those which presented the most pronounced opalescence. Active filtrates, which at first inspection appeared to be clear or to give a faint Tyndall effect, were found to yield a sediment which, after washing, made an opalescent solution. Such solutions carried most of the tumor-producing activity of the filtrate. An estimation of the degree of dependence between the tumor-producing activity and the power of the solution to scatter light is prevented by the existence of two factors, essentially variable; namely, the concentration of inhibitor and the proportion of the agent in the inactivated form. The same considerations apply to the study of the material examined with the ultramicroscope. Seen in the dark field microscope (dark field condenser and carbon arc lamp), the solutions carrying the activity appeared to contain innumerable particles of approximately, but not exactly, the same size. These particles appeared as minute granules, most of them isolated or in aggregates of several units. The biggest particles formed agglomerates suggesting microscopic clusters of precipitated proteins. All these particles showed the brownian movement.

DISCUSSION

The results presented in the present paper indicate that the agent causing Chicken Tumor I can be concentrated and, to a certain extent, purified by means of high speed and low speed centrifugation. The fraction, prepared by this method, represented about 90 per cent of the total tumor-producing activity and 0.035 per cent of the total solids of the original filtrate. On the basis of dry weight, the tumor agent was concentrated about 2,800 times.

Most of the tumor agent was deposited when the tumor filtrate was submitted to a centrifugal force of 14,000 times gravity for 1 to 4

hours.   The persistence of some activity in the filtrate even after prolonged centrifugation might be attributed to disturbing forces, such as vibration or convection currents, which would interfere with a proper separation, or to redispersion of part of the sediment during rapid deceleration.   In the present work, however, these factors were not sufficient to affect appreciably the separation from solution of *Helix* and *Limulus* respiratory proteins, used as control under the same conditions.   The possibility that the tumor agent may occur in different states of aggregation or dispersion in the tumor extract is not completely excluded.   Svedberg showed that native proteins may coexist in solution together with several aggregation or dissociation products of well defined molecular weight.   The proportion of the various molecular species present may be conditioned by dilution or by the pH of the solution, and, within certain limits, the process is a reversible one (7).   Erikson-Quensel and Svedberg suggested that the mosaic virus protein may exist as a monodisperse system in the native state, but they have shown that inhomogeneity can easily be brought about *in vitro* (8).   Whether a portion of the extracted tumor agent may also dissociate into particles of smaller size, which would require a greater centrifugal force for their sedimentation, will be tested in further experiments.

The speed of sedimentation of the tumor agent, or that part of it which was completely sedimented by a centrifugal force of 14,000 times gravity, is in fair agreement with the expected velocity calculated for a particle of 70 mμ diameter, a figure estimated by Elford and Andrewes (9) to represent the probable size of the tumor agent, and for a density of 1.3.   Centrifugation at that speed for 30 to 45 minutes was sufficient to throw down the agent, when the medium was Tyrode's solution, 0.9 per cent salt solution, or water.   Under the same conditions complete sedimentation of the respiratory pigment of the snail, with a sedimentation constant of 98.9×10^{-13}, required about 8 times longer.   The diameter of the *Helix* hemocyanin molecule, calculated by Svedberg and Chirnoaga from sedimentation measurements, is equal to 24 mμ (5).   Assuming the particles to be spherical, the volume of the tumor agent (radius $= 3.5 \times 10^{-6}$) and of *Helix* hemocyanin (radius $= 1.2 \times 10^{-6}$) would be 1.79×10^{-16} and 7.24×10^{-18} cc., respectively.   According to these figures, the tumor agent would correspond to 24.7 molecules of *Helix* hemocyanin by

volume. For a density of 1.36 and 1.3, respectively, the molecular weight of the particle would be 5.9 millions for the *Helix* hemocyanin molecule and 140 millions for the chicken tumor agent. Whether a particle of this size is chemically uniform or represents a system of more than one chemical substance remains to be determined. In a recent study it was shown that the tumor agent was inactivated by crystalline trypsin, indicating that a protein is an essential constituent of the particle (10).

The experiments here presented have shown that the total tumor-producing activity of the filtrate was increased through the process of centrifugation and washing of the sediment. Recent experiments (11) have shown that an active agent and an inhibiting factor, associated in inactive extracts of a chicken fibrosarcoma (Chicken Tumor 10), could be separated by means of high speed centrifugation. These observations support the view that the enhanced activity of the material separated from Chicken Tumor I extracts is the result chiefly of the dissociation of the tumor agent from an inhibitor. That such inhibitor exists in Chicken Tumor I was demonstrated in this laboratory, and its separation was accomplished by various methods. Extracts from fast growing tumors were more active after being treated with an adsorbing agent, which removed the major part of the proteins in solution; and tumor desiccates yielded more active preparations after serial extraction (12). The present results are in agreement with these findings. The evidence that the tumor agent can be separated from the inhibitor by physical methods suggests that the two agents may fail to react and remain independent in mixtures that are inactive. In other words, these observations seem to indicate that the suppression of the tumor-producing activity of an extract is not the result of a direct neutralization of the active agent, and can be effective without destruction of the agent itself.

SUMMARY

1. The agent causing Chicken Tumor I can be separated from the other constituents of the tumor filtrate by means of high speed centrifugation. The separation was practically complete when a filtrate of average viscosity (0.018 poise) was submitted to a centrifugal field of 14,000 times gravity for 2 hours.

2. Relative purification of the agent was obtained by means of

differential centrifugation and washing in Tyrode's solution or in distilled water. The washed sediment gave opalescent solutions composed of minute particles of approximately, but not exactly, the same size. The dry weight of the active material separated by high speed centrifugation was 0.0008 mg. per cc. of filtrate, or about 1 part per 2800 parts of the total filtrate.

3. The tumor-producing activity of the washed sediment was significantly greater than that of the entire original filtrate. It is suggested that the gain in tumor-producing power was effected by the removal of an inhibiting factor, known to occur normally in chicken tumor extracts.

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LYMPH NODES AS A SOURCE OF NEUTRALIZING PRINCIPLE FOR VACCINIA

By PHILIP D. McMASTER, M.D., and JOHN G. KIDD, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, March 9, 1937)

Previous papers from this laboratory have shown that every intradermal injection is truly intralymphatic (1–3). Dyes introduced in this way enter the superficial plexus of lymphatics through channels torn by the needle; and particulate substances which are punctured, scratched or even "vaccinated" into the skin pass in some part directly into these channels and are carried to the regional nodes (4, 5). Torn lymphatic capillaries remain open for hours (5), whence it follows that infectious agents introduced by way of a skin wound may reach the lymphatic glands directly. All this being the case, the possibility suggests itself that lymph nodes may do more in defense of the body than act as barriers in the way of invading organisms. Their activity in the latter relation is attested by their secondary involvement in infections of the skin and mucous membranes. Recent work has demonstrated the formation of specific bacterial agglutinins in lymph nodes draining the ears of mice into which killed cultures of bacteria had been injected intradermally (4). Experiments have now been carried out to determine whether the lymph nodes of rabbits elaborate a neutralizing principle effective against vaccine virus draining to them.

Methods and Preliminary Experiments

The lymphatics of the rabbit's ear converge into one or two trunks which drain into a large lymph node in the region of the parotid gland and into a group of 2 or 3 nodes situated at the junction of the internal and external jugular veins. India ink and vital dyes can be injected intradermally in the outer fourth of the rabbit's ear in such a way that several lymphatics are invariably torn by the needle, with the consequence that some part of the injected material drains to these nodes. Furthermore, it seems probable that much of the dye lying intersti-

tially after such an injection, later passes along the same channels (4). Dissections of the head and neck of 4 rabbits that had been injected into the ear with 0.05 to 0.4 cc. of dye intradermally showed that the colored fluid always reached the nodes in a few minutes. On the basis of this knowledge an experiment was carried out as follows:

A suspension of vaccine virus, prepared as described below, was faintly colored by adding to each 50 cc., 0.3 cc. of an isotonic 11 per cent aqueous solution of an innocuous vital dye, patent blue V[1] (1, 2). 0.1 cc. of the colored suspension was injected intradermally in the outer fourth of the left ears of 3 rabbits. Almost at once the lymphatics at the base of the ear became visible because of a content of stained fluid. 2 hours later, to prevent further possible drainage to the nodes of the injected side, the ear on this side was amputated under ether, and so too was its fellow. One of the animals was killed at once, the others after 24 and 48 hours. Dissection showed the draining lymphatics in the neck and the parotid and cervical lymph nodes to be very faintly colored with dye in each instance. The left cervical lymph nodes of the two animals killed last were enlarged 4-fold, pale blue and hemorrhagic, whereas the right parotid and cervical nodes appeared normal. All were removed with precautions for asepsis, extracted by a method to be detailed shortly, and the extracts injected into the shaved skin of normal rabbits. In each instance extracts of the nodes from the side injected with vaccine virus yielded typical vaccinia lesions, whereas those from the uninjected side gave none.

Having determined in this way that vaccinia entering the peripheral lymphatics can be demonstrated in the regional lymph nodes 48 hours later, we next sought evidence of its fate within the glands. Does it increase or decrease in the nodes with the passage of time? Do antiviral principles appear in the nodes? And if so, are these formed within them?

To answer some of these questions experiments were begun involving the injection of suspensions of vaccine virus and its recovery from various tissues or body fluids as well as its subsequent titration. The techniques used will be described before detailing the experiments.

Preparation of Vaccine Virus Suspensions.—Vaccine virus of the New York City Board of Health strain was provided[2] as a 28th subculture with chick embryo tissue and Tyrode's solution, cultivated *in vitro*. The pathogenicity of the strain had been revived twice by inoculation into susceptible rabbits (6) and it was propa-

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[1] General Dyestuff Corporation.
[2] This virus was kindly provided by Dr. T. M. Rivers.

gated further by us in the testicles of rabbits. For the purposes of this work the testicles of one rabbit, 3 days after inoculation, were minced with scissors, well mixed and passed through a Latapie grinder. 1 gm. portions were sealed in small tubes and placed in a freezing chamber at $-20°$ to $-22°C$. Later, when needed, active virus suspensions were obtained by grinding the contents of one of these tubes with sand in a TenBroeck grinder, diluting with 100 cc. of Tyrode's solution, and centrifuging at 1800 R.P.M. for 10 minutes. The supernatant fluid was transferred to small tubes and again centrifuged at 2500 to 3000 R.P.M. for 20 minutes. To exclude cells that might conceivably still be living (7), a monel metal disc, attached to a stout wire and heated red-hot was held 2 or 3 mm. above the level of the fluid in the tubes until the surface layer boiled, thus killing such cells as might have come to the surface. The clear fluid of the middle layer was aspirated into a sterile syringe through a long lumbar puncture needle which was not allowed to touch the glass. The needle was removed and the aspirated fluid expelled into a fresh tube. A separate syringe and needle were used for each aspiration. The clear fluids thus obtained were centrifuged at high speed and again "de-celled" in the same way. On inspection under the microscope the 1 per cent suspension obtained in this way appeared cell-free. It was employed as such or diluted 10-fold before injection.

Methods of Inoculation.—In about half the instances, 0.3 to 0.4 cc. of the 1 per cent virus suspension was injected intradermally, 0.05 to 0.1 cc. at each of 4 sites near the shaved tip of the ear. Before expelling the fluid, the injecting needle was thrust 3 or 4 times into each area to tear many lymphatics, thus assuring the introduction of some of the inoculum and the consequent transport of virus to the cervical lymph nodes. Under ether anesthesia the injected ears were amputated at the base, that is to say several centimeters from the nearest injection site, 2 to 4 hours later. This was done to prevent the development of large vaccinia lesions at the site of injection, with consequent drainage of much virus to the nodes. In most instances the ear stumps healed without evidence of vaccinia infection there, but in a few animals local lesions developed. Because of this, other experiments were done without ear amputation, but with the dose of virus greatly reduced. In these instances the original 1 per cent virus suspension was diluted 10-fold, and only 0.1 cc. injected, at one site, that is to say approximately 1/40 of the dose used in the other experiments. Rabbits so injected developed typical vaccinia lesions at the injection sites, and no doubt virus drained to the neighboring lymph nodes during a period of several days.

In all of the animals virus was injected into one ear only, with the addition in the earlier experiments of the small amount of dye already described. The dye served to demonstrate, not only that the fluid entered the lymphatics at the time of injection, but also showed at autopsy to which of the lymph nodes it had come. In later experiments the use of dye was abandoned because we had found that virus never failed to appear in the chain of lymph nodes draining the injected side. The omission did not entail any differences in the findings.

As result of the virus injections the cervical lymph nodes on the injected side became enlarged, inflamed and often hemorrhagic. In all the experiments the cervical nodes from the uninjected side as well as nodes from other portions of the body served as controls. To control the possibility that the inflamed nodes might in some manner take up from the blood protective principles formed elsewhere in the body, the cervical nodes of the side not injected with virus were experimentally inflamed in more than half the instances. This was done by injecting into the ear of that side New York City Board of Health typhoid bacterin containing 1000 million killed *B. typhosus* organisms to the cubic centimeter together with 750 million of paratyphoid α and β. The bacterin was diluted 4-fold with Tyrode's solution colored, as was the virus suspension, with patent blue V, and injected intradermally in the ear in the same amounts as the virus suspension in the other ear. The bacterin caused the cervical lymph nodes to become enlarged, edematous and inflamed, the gross changes in these respects being roughly equivalent to those on the virus-injected side. They were tinged pale blue by the dye.

In many experiments the injection of typhoid bacterin on the control side was omitted in order to compare the size of approximately normal lymph nodes with that of the enlarged ones on the virus-injected side. This was done only after several experiments had shown that virus could not be demonstrated in the nodes of the typhoid-injected side even after they had become inflamed, as further that extraction of them failed to yield a neutralizing fluid for vaccinia until many days after this had become the case with the corresponding nodes of the virus-injected side.

At varying intervals from a few minutes to 15 days after introduction of the virus, the injected animals were etherized and samples of blood obtained aseptically for serum. The organs for study were then removed with aseptic technique. The lymph nodes from the parotid region and those lying at the junction of the internal and external jugular veins of the control side were first removed and weighed as a group. They will be termed the cervical nodes. The axillary nodes and one large inguinal gland were then taken from the same side and, if the experiments required it, samples of femur or tibia marrow, spleen or liver. To avoid possible contamination of other tissues with virus the cervical lymph nodes of the virus-injected side were removed last. All these tissues were weighed, placed in small Petri dishes and kept moist with filter paper saturated with Tyrode's solution. After tracings had been made of the size of the nodes the dishes were put in the freezing chamber at $-20°$ to $-22°C.$ and 1 to 10 days later the frozen material was ground aseptically with sand, and Tyrode's or Locke's solution added to form 10 per cent suspensions. These were cleared in the centrifuge at 2500 R.P.M. for 10 minutes and the supernatant fluids were then tested.

Presence of the Virus.—To determine the presence of the virus in the extracts, they were diluted in multiples of 10 to 10^{-5} or in some experiments to 10^{-7}. Node material from the control side was diluted in this way but only to 10^{-1}, 10^{-2} and 10^{-3}. The diluted suspensions were then inoculated intradermally (0.2 cc.) into the shaved sides of at least 3 normal brown-gray rabbits, varying the site of in-

jection from animal to animal. In each of these animals vaccine virus was inoculated as well, using for the purpose a freshly made de-celled 1 per cent suspension diluted to 10^{-5}, 10^{-6} and 10^{-7}. This was obtained from another frozen sample of the same testicular material originally employed for the ear injections nnd kept at $-30°C$. in the freezing chamber, like the original material before its injection.

Neutralization Tests.—These tests were employed to demonstrate the presence of antiviral principles in the serum and in the tissue extracts. Some of the freshly made 1 per cent vaccine virus suspension was diluted 50,000, 500,000 and 5,000,000 times. The resulting fluids were mixed in equal parts with the cleared, centrifuged 10 per cent tissue extracts or with whole serum or serum diluted 1 in 10 with Locke's solution, or both. There resulted 5 per cent tissue or serum mixtures with vaccine virus in dilutions of 10^{-5}, 10^{-6} and 10^{-7}. These mixtures were allowed to stand at room temperature for 1 to 3 hours before they were employed. The differences in interval caused no observable difference in the findings. This was to have been expected from the work of Andrewes (8), who found that immune serum incubated with vaccine virus before testing (within the limits imposed by the tendency of the virus to deteriorate) showed no better neutralization upon inoculation into animals than did freshly made mixtures. Intradermal inoculations of 0.2 cc. of each of the virus-extract mixtures were then made upon at least 3 rabbits which also received the plain vaccine virus suspensions on the sides. Daily, for 7 days, these animals were examined and the size and character of the lesions traced and noted.

The Fate of Vaccine Virus in the Lymph Nodes.

How long does vaccinia persist in the cervical lymph nodes following intradermal inoculation in the ear?

An experiment to answer this question disclosed outstanding differences in the amount of virus present in the cervical lymph nodes of the injected side in animals which were allowed to survive for differing periods of time after amputation of the ears.

In the manner already described, vaccine virus suspension, prepared in the usual manner, was injected near the tip of the shaved left ears of 6 rabbits. Under ether anesthesia these ears were amputated 4 hours later. After another hour one of the animals was killed and the cervical lymph nodes on the injected and uninjected sides were separately removed and placed in the freezing chamber. On the next day and upon the 3rd, 5th, 7th and 11th days, respectively, n single animal of the lot was sacrificed and the material was treated in the same way.

The de-celled extracts of cervical nodes in dilutions of 10^{-1} to 10^{-5} with Tyrode's solution were then intradermally injected into the shaved sides of 3 brown-gray rabbits. The sites of injection were varied as usual. Only the extracts of the cervical nodes from the virus-injected side yielded lesions. The more concentrated
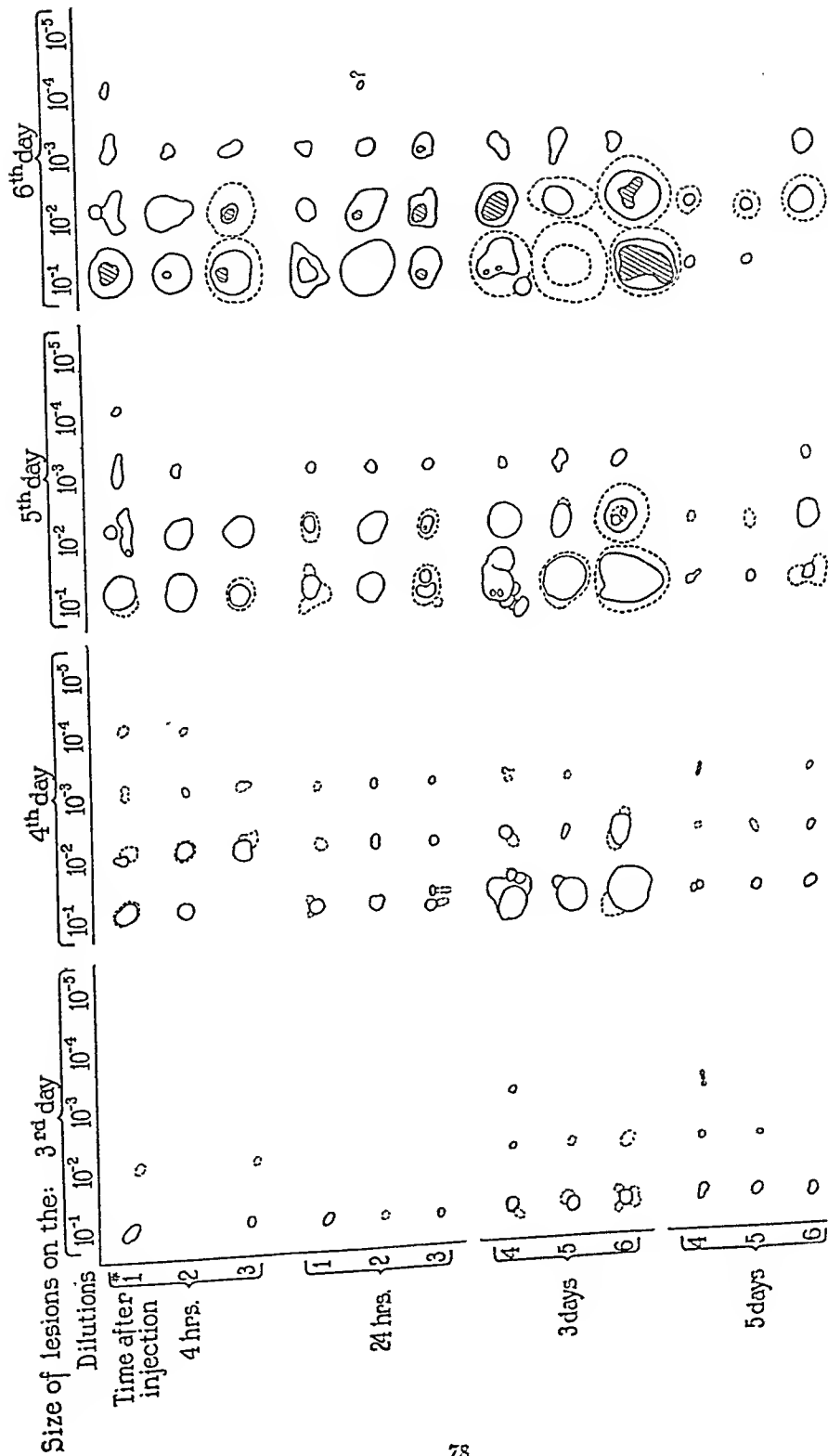
TEXT-FIG. 1 a. The vaccine virus content of cervical lymph nodes at intervals after injection in the ear.

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Size of lesions on the: 3rd day

Dilutions 10^{-4} 10^{-5} 10^{-6} 10^{-7}

7

8

TEXT-FIG. 1. Lesions produced by vaccine virus in Tyrode's solution for comparison.
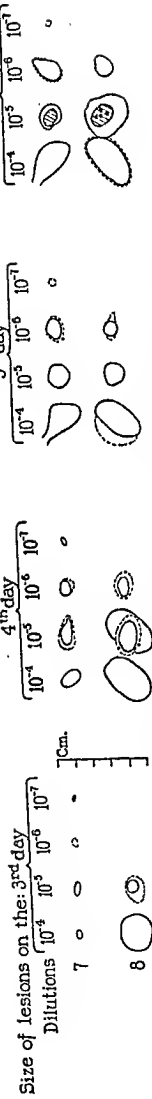
TEXT-FIG. 1 b. Lesions produced by vaccine virus in Tyrode's solution for comparison.

TEXT-FIG. 1. Successive tracings of vaccinia lesions appearing on the shaved sides of 3 normal rabbits intradermally injected, as described in the text, 4 hours, 1 day, 3 days and 5 days previously with equal amounts of the extracts of the cervical lymph nodes of other rabbits which had been inoculated with vaccine virus in one ear and typhoid vaccine in the other. Only the node extracts from the nodes draining the virus-injected side gave lesions. The lesions developing from extracts removed 4 and 24 hours after injecting the ear were equal in size. Lymph node material taken on the 3rd day after virus injection in the ear gave larger lesions, that removed on the 5th day smaller ones. The significance of this is discussed in the text. For comparison, tracings of control lesions produced in the same animals by injections of virus suspensions are shown. The continuous lines represent the boundary of the lesion itself, the dotted lines the surrounding edematous swelling. The hatched central areas represent beginning necrosis, the cross-hatching, advanced necrosis, the solid block areas, crater formation in regressing lesions. All tracings have been reduced to the same extent.

Text-figs. 4 to 9 show tracings of vaccinia lesions of test animals in the other neutralization tests described in the text. The findings are self-explanatory, when studied in conjunction with the text.

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extracts developed characteristic vaccinia lesions by the 3rd day, the more dilute extracts 24 to 48 hours later. These ran the characteristic course and daily for a week their size was recorded, either by tracing it on a piece of transparent celluloid or by transferring caliper measurements to paper. The tracings taken from the test animals on the 3rd, 4th and 5th days are shown in Text-fig. 1. In this and in subsequent charts the continuous lines represent the raised, indurated border of the lesion itself. The dotted lines show the boundary of the edematous swelling about the lesion, when present. Areas of beginning necrosis have been shown by hatching, advanced necrosis by cross-hatching, and crater formation in regressing lesions by solid black areas. The tracings in all the text-figures have been reduced to the same extent and are therefore comparable.

As shown in Text-fig. 1 the dilutions of the lymph node extracts recovered 4 and 24 hours after injecting rabbits in the ear with vaccine virus gave rise to similar lesions. Material from the animals killed on the 3rd day yielded larger lesions; that obtained 5 days after virus injection yielded smaller lesions again, and only in the lower dilutions. After 7 days still smaller lesions were obtained and the material taken on the 11th day yielded practically none. The findings from the last 2 animals have not been included in Text-fig. 1, since they obtain abundant illustration in the charts of subsequent experiments.

For about 3 days after the virus had been injected intradermally in the ears of the rabbits of this experiment, it appeared to increase within the cervical lymph nodes, as Text-fig. 1 shows, and this although the injected ears had been amputated 4 hours after the injection. By the 5th day less virus was demonstrable, by the 7th day still less, and on the 11th day, none.

It is evident from the survey of the technique already given that the interpretation of our results depended upon the finding of differing amounts of virus or of protective principle in the corresponding lymph nodes of the two sides. We desired to know whether the various amounts of virus found in the lymph node extracts could be ascribed to differences in the amounts which had reached the cervical lymph nodes immediately after intradermal injection into the ear. An experiment was done to test the point.

Near the shaved tip of the left ear of each of 4 normal brown-gray rabbits 4 intradermal injections were made into the same approximate situations, of 0.1 cc. each of freshly de-celled 1 per cent vaccine virus suspension. In the right ears typhoid bacterin was similarly injected at 4 sites, 0.1 cc. in each. Approximately 4 hours later the left ears of these animals were amputated, under ether, to render the technique comparable with that of other experiments to be reported below. They were then killed with chloroform and the right and left cervical lymph nodes

were aseptically removed and separately extracted with Locke's solution as already described. Dilutions of the extracts, to 10^{-1} to 10^{-5}, were then injected separately in amounts of 0.2 cc. intradermally into the shaved sides of 3 normal brown-gray rabbits.

In the meantime the left ears of 2 other animals were injected but once, with 0.1 cc. of the virus suspension diluted 10-fold and the right ears injected with typhoid bacterin as described above. The ears were not removed later. The following day these 2 rabbits were killed and similar titrations made with extracts of the cervical lymph nodes. The lesions resulting from both sets of titrations were charted daily for 7 days. No growth resulted from the fluids obtained from the right nodes, the side injected with typhoid bacterin. The left node material of all the rabbits yielded typical vaccinia lesions at dilutions of 10^{-1}, 10^{-2} and 10^{-3} in all instances and at 10^{-4} in only one.

Only slight differences appeared among the lesions developing from the node extracts taken from the 4 animals with amputated ears. In size and in rate of development the lesions were like those yielded in the preceding experiment by node extracts made 4 and 24 hours after injecting virus in the ear. (Tracings in the first two rows of Text-fig. 1.) The differences were in no way as great as those shown in the remaining portions of Text-fig. 1. Obviously the cervical lymph nodes of the 4 rabbits contained similar amounts of virus 4 hours after the injection. Differences in the virus content of cervical lymph nodes as obtained in the previous experiment and in those to be described below may therefore be attributed to changes in the amount of virus after it had reached the nodes. The titration tests for the 2 rabbits given small doses of vaccine virus and allowed to live for 24 hours showed also no differences, indicating that similar quantities of virus reached the nodes in both animals. By chance the lesions were strikingly similar to those of the previous titration tests. Similar amounts of virus were present in the cervical nodes of the two groups of animals, one 24 hours after allowing a small dose of virus to drain in an intact ear, the other 4 hours after making the larger injection.

In the preceding experiment (of Text-fig. 1) the virus content of the cervical lymph nodes was greatest in the animal injected 3 days previously. Nodes removed on the 5th and 7th days yielded less and less virus and by the 11th day gave almost no evidence of its presence. Was the virus destroyed within the nodes, and if so, how? To answer this question neutralization tests were done, to compare the lesions produced in the skin of healthy rabbits, by vaccine virus at certain Tyrode dilutions, with those resulting when it had been diluted instead with extracts of lymph nodes to which vaccinia had been carried on the lymph some days before. But first we determined the effect of extracts of normal lymph nodes.

Duran-Reynals (9) has shown that certain tissue extracts, notably those of the testicle, enhance the lesions produced by vaccine virus, whereas other extracts inhibit it. He found the effect of lymph node extract to be very slightly inhibitory (9). It seemed wise to make further tests under the conditions of our experiments. They were carried out as a matter of general information despite the fact that we employed in all of our subsequent experiments control mixtures of vaccine virus and of extracts of lymph nodes from the side not injected with virus.

The cervical lymph nodes were removed aseptically from a chloroformed normal rabbit, and 10 per cent extracts were made in the usual manner. A freshly prepared, de-celled vaccine virus suspension was mixed with equal parts of the lymph node suspensions to yield final dilutions of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}. Each of these fluids was injected intradermally (0.2 cc.) at 2 sites in the shaved skin of each of 3 normal brown-gray rabbits. The resulting lesions were observed and traced daily for 10 days. For the sake of brevity no charts will be given, since in the charts of subsequent experiments comparable tracings are shown of the lesions arising from inoculations of plain virus and of virus mixed with extracts of normal and pathological lymph nodes. The extracts of normal lymph nodes exerted only the slightest inhibitory action, if any, upon the development of vaccine virus lesions, as shown by a comparison with the control lesions caused by plain virus at similar dilutions.

Before searching for antiviral principles in the lymph nodes it was necessary to cope with one other possibility. It is well known that antibodies can be demonstrated in many organs and body fluids. In an animal flooded with antigen, they may appear in such quick succession in the various tissues and body fluids that one is unable to determine in which they first occurred. The satisfactory demonstration of the presence of a neutralizing principle for vaccine virus in one tissue or body fluid prior to its appearance elsewhere in the body, after injection in the ear, called for a dose of the virus so gauged that the rapid development of generalized vaccinia did not happen, although the animals became immune. We determined the dose by observing the clinical course of the disease for 10 days in rabbits injected in one ear with various amounts of the virus. The temperature of each animal was noted twice daily and the shaved skin of both sides of the body was watched for the appearance of pocks. Furthermore sterile testicular stabs were made, as hereafter described, with a view to inducing localization of vaccinia which would disclose its presence in the circulation. It is well known that generalized skin pocks develop in rabbits receiving large doses of vaccine virus intra-

dermally. We have noted the almost invariable appearance of skin pocks, usually on the 5th or 6th day after inoculation in our titration rabbits which necessarily received large amounts of virus. We therefore examined the rabbits injected in the ear with small doses of virus for a period of 7 days to see if they too developed generalized pocks. The final experiment to ascertain the dose selected for the purposes of the present work will be briefly described.

Six normal brown-gray rabbits were injected in one ear with 0.1 cc. of fresh vaccine virus suspension diluted to 10^{-2}. 4 hours later both ears were amputated

°F.
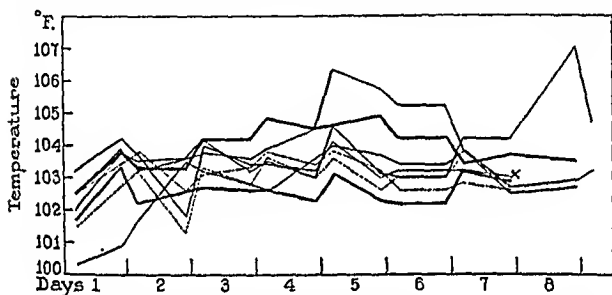
TEXT-FIG. 2. Temperature curves of 6 rabbits injected in one ear with 0.1 cc. of a fresh vaccine virus suspension at a dilution of 10^{-2}. The two heavy continuous lines record the temperatures of 2 of these animals in which, as described in the text, sterile testicular stabs were made. No localized vaccinia lesions developed in these. The broken lines are the temperature curves of 2 uninoculated control rabbits. None of the animals receiving the virus developed generalized vaccinia.

near the base. Rectal temperatures were taken at 10 a.m. and 4 p.m. daily. The following day a wide area was shaved over the abdomen and both sides of each animal, not only to facilitate the finding of pocks should they develop but to cause abrasions and slight injuries of the skin in which virus might become localized. Under ether, aseptic testicular stabs were made in 2 of the rabbits, using 19 gauge needles. The organs were examined daily thereafter for evidence of lesions. On the 2nd, 3rd and 4th days the ear stumps showed only a slight amount of soft edema; none was red, warm or inflamed. Subsequent work has shown that the virus can be obtained from such stumps. On the 5th day the stump of the injected ear of one rabbit showed an irregular area of dry necrosis 1 cm. in diameter overlying the central artery and lymphatic trunks. Surrounding the necrosis

brawny induration extended for 2 cm.   At no time was this animal's temperature
markedly elevated (Text-fig. 2, the line marked with an x), nor did the abdominal
area show pocks.   In later experiments about one-third of the animals so injected
showed vaccinia lesions in the ear stumps.   No pocks developed on the shaved
areas of skin.   In Text-fig. 2 the temperature curves of these animals are plotted.
There was no exceptional increase in the animals with testicular stabs (heavy
lines) and no localized lesions developed.   It will be seen that the rabbits injected
in this way failed to show the clinical signs of generalized vaccinia.   Text-fig. 3
shows as contrast the temperature curves (for the first 4 days only) of 3 rabbits
used for routine propagation of the virus, and which received 1 intratesticular
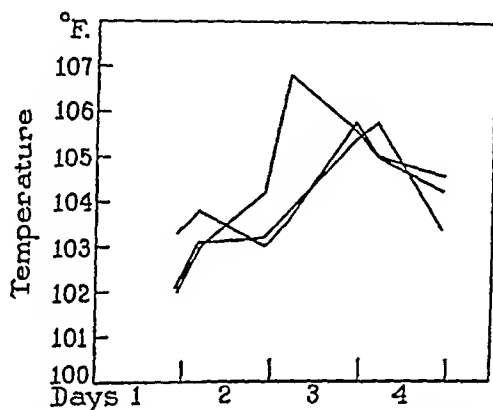
TEXT-FIG. 3.   The temperature curves of 3 rabbits inoculated intratesticularly
with 0.75 cc. of a 1 per cent suspension of the same virus-containing material used
with the animals of Text-fig. 2.   Generalized vaccinia developed.

injection of 0.75 cc. of a 1 per cent suspension of the same virus-containing ma-
terial used to inoculate the animals charted in Text-fig. 2.   These animals ex-
hibited generalized pocks.

On the 7th day after inoculation 2 of the group of experimental rabbits were
killed and the cervical lymph nodes of the injected and intact side were removed,
as also samples of spleen, bone marrow and serum.   Extracts of these tissues and
specimens of the blood serum as well were utilized in titration and neutralization
tests.   The latter disclosed the presence of a neutralizing principle in the serum
and in the extracts of the cervical lymph nodes of the virus-injected side, as will
be evidenced further on.   The animals that were let live and others similarly in-
jected in later experiments proved refractory to infection when reinoculated with
0.2 cc. of the 1 per cent vaccine virus suspensions 2 weeks after the original in-
jection.

The amount of vaccinia virus employed in this experiment gave rise to a localized infection which resulted in generalized immunity. In the later experiments the same dosage was employed.

The Local Development of an Antiviral Principle

The foregoing results provided a basis for neutralization tests to determine whether or not the lymph nodes are capable of elaborating an antiviral principle. At various time intervals serum, cervical and other lymph nodes and portions of the bone marrow and spleen were procured from 36 rabbits and titration and neutralization tests were carried out. In 6 instances the materials were taken 4 to 6 hours after inoculation of vaccine virus into the ear, in another instance after 24 hours, in 1 after 2 days, in 5 after 3 days, from 6 animals upon the 4th day, from 6 on the 5th day, 2 on the 6th day, 4 on the 7th day, 2 on the 11th and 3 on the 15th day. The outcome of these experiments is best shown by presenting first the findings in the animals that had become highly resistant to vaccinia 15 and 11 days after inoculation and then describing the results obtained from animals showing less and less neutralizing principle in the serum and organ extracts, 7, 6, 5, 4 and 3 days after inoculation, respectively.

15 days after the inoculation of virus in the left ears of 7 rabbits in the manner described, 3 were killed. Specimens of the serum were taken and extracts made of the cervical lymph nodes from both sides, separately, from axillary and inguinal lymph nodes, bone marrow and spleen. Titration tests and neutralization tests were carried out on 3 normal brown-gray rabbits, varying the injection sites as usual. The titration tests, even those done with the extracts of cervical lymph nodes from the virus-injected side, yielded no lesions. The neutralization tests, employing the extracts mentioned above mixed with vaccine virus suspension diluted 10^{-5}, 10^{-6} and 10^{-7}, showed either complete neutralization, or marked inhibition of the ability of the virus to form lesions. In Text-fig. 4 are given the tracings of the few scanty lesions which appeared in these tests. Tracings of other lesions produced in the same test animals by suspension of virus alone are shown for comparison. It is to be noted that no lesions appeared when virus was mixed at a dilution of 10^{-5} with the extract from the lymph nodes of the virus-injected side or with the serum. Obviously an antiviral principle was widely present in the animals.

The remaining 4 rabbits inoculated 15 days previously were intradermally injected in their shaved sides with 1000 infective doses of our virus and were found to be refractory. They served as controls to the state of the 3 animals which were killed to furnish material for the titration and neutralization tests.
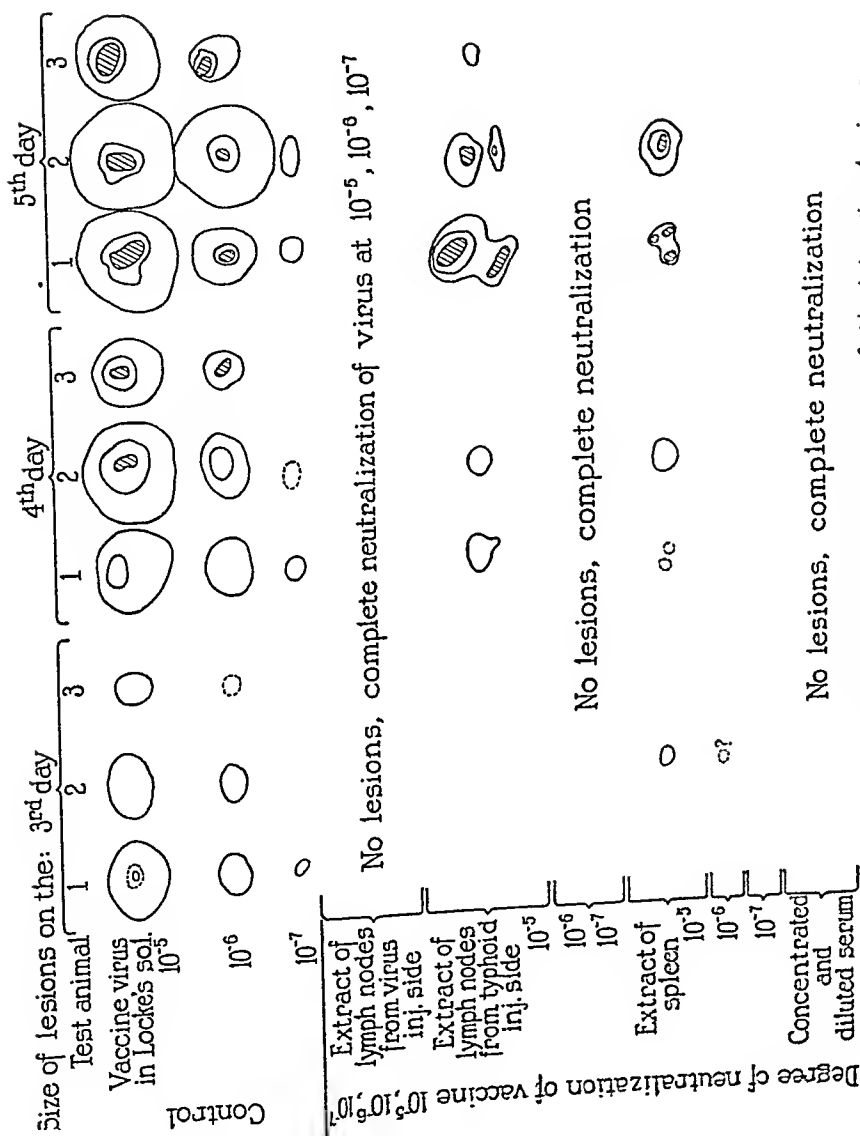
TEXT-FIG. 4. Neutralization tests with tissue extracts and serum. Material procured after 15 days.

In another experiment 2 rabbits were inoculated with vaccinia in one ear and with typhoid bacterin in the other, as described. On the 11th day thereafter, titration tests with serum, and the usual lymph node, spleen and marrow extracts failed to yield evidence of the virus. Neutralization tests showed neutralization of the virus almost equal to that after 15 days. Obviously in these animals too the antiviral principle was widespread in the body and very effective.

Four rabbits injected 7 days previously with virus in one ear and with typhoid bacterin in the other served as the source for serum and tissue extracts procured in the usual way. In 2 of these instances the ears were amputated, in the others left intact. Each extract was titrated upon 3 rabbits and neutralization tests carried out on 3 more with each of the extracts against vaccine virus at dilutions of 10^{-5}, 10^{-6} and 10^{-7}, varying the injection sites. The findings in all were similar. Text-figs. 5 and 6 of typical instances show the results of the neutralization tests with virus at dilutions of 10^{-5} and 10^{-6}, as they appear in the test animals on the 3rd, 4th and 5th days after inoculation. In the instance used for Text-fig. 5 the serum was diluted 1 in 10, that is to say to the same extent as the tissues from which the extracts were made. In the instance shown in Text-fig. 6 whole serum was used.

7 days after inoculation of virus in one ear and typhoid bacterin in the other the serum and all the tissue extracts showed some neutralizing ability, but far less than that of similar material taken from animals 11 days after inoculation. The highest concentration of antiviral principle was found in whole serum and in the extracts of the cervical lymph nodes of the injected side. Serum, diluted to the same extent as the lymph node tissue, and spleen extract exhibited less effectiveness in an almost equal degree. As Text-fig. 5 shows the extract of the lymph nodes of the typhoid-injected side showed least neutralizing power. By titration tests, not shown in the chart, virus was demonstrated to be still present in the nodes of the virus-injected side but there only.

Text-fig. 6 demonstrates the strong neutralizing power of whole serum 7 days after inoculation in the ear, as compared with that of the diluted serum (Text-fig. 5). In making this comparison it is to be recalled that the lymph node tissue was diluted 10-fold and a direct comparison of its neutralizing power with that of whole serum cannot be made. One can only say that whole serum neutralized virus more readily than did the diluted lymph node extract. The neutralizing power of serum procured on the 7th day is not found in animals sacrificed earlier, for example on the 4th day, and the results in Text-fig. 6 should be compared with those in Text-figs. 8 and 9 in which the neutralizing effect by extract of the lymph nodes from the injected side proved to be greater than that of whole serum. The significance of this will be discussed below.

Two similar experiments were done, using material from animals sacrificed 6 days after inoculation of virus in one ear, in the usual manner. Text-fig. 7 shows the results of the neutralization tests of one of these instances. Vaccine virus suspension at 10^{-5} and 10^{-6} was much inhibited by the extract of lymph nodes
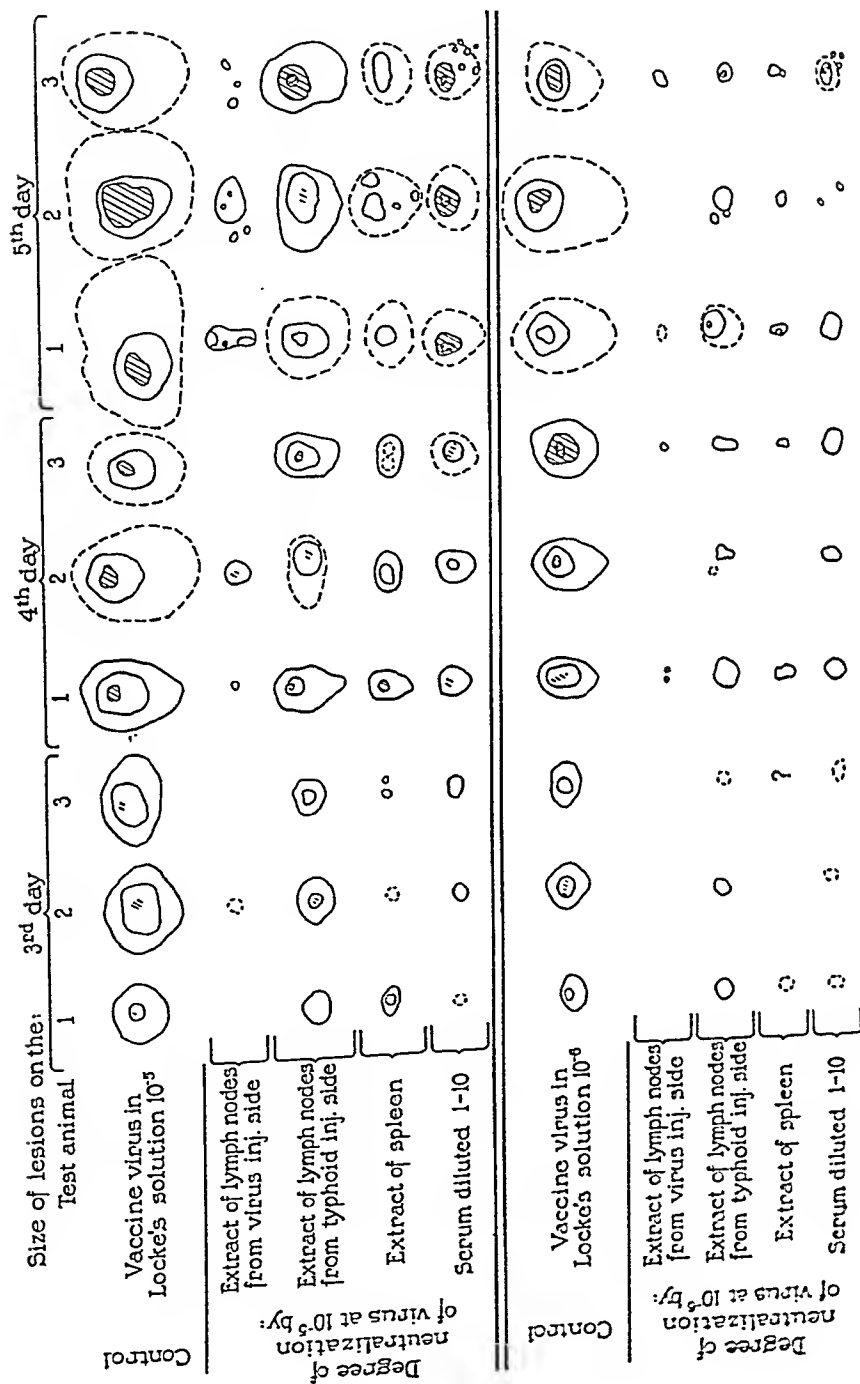
TEXT-FIG. 5. Neutralization tests with tissue extracts and serum. Material procured after 7 days.
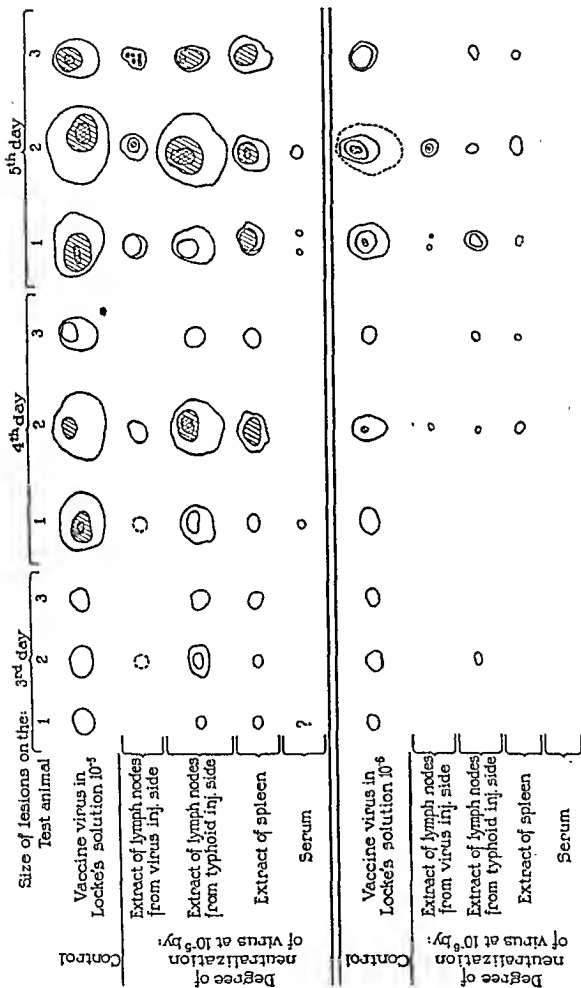
Text-Fig. 6. Neutralization tests with tissue extracts and serum. Material procured after 7 days.
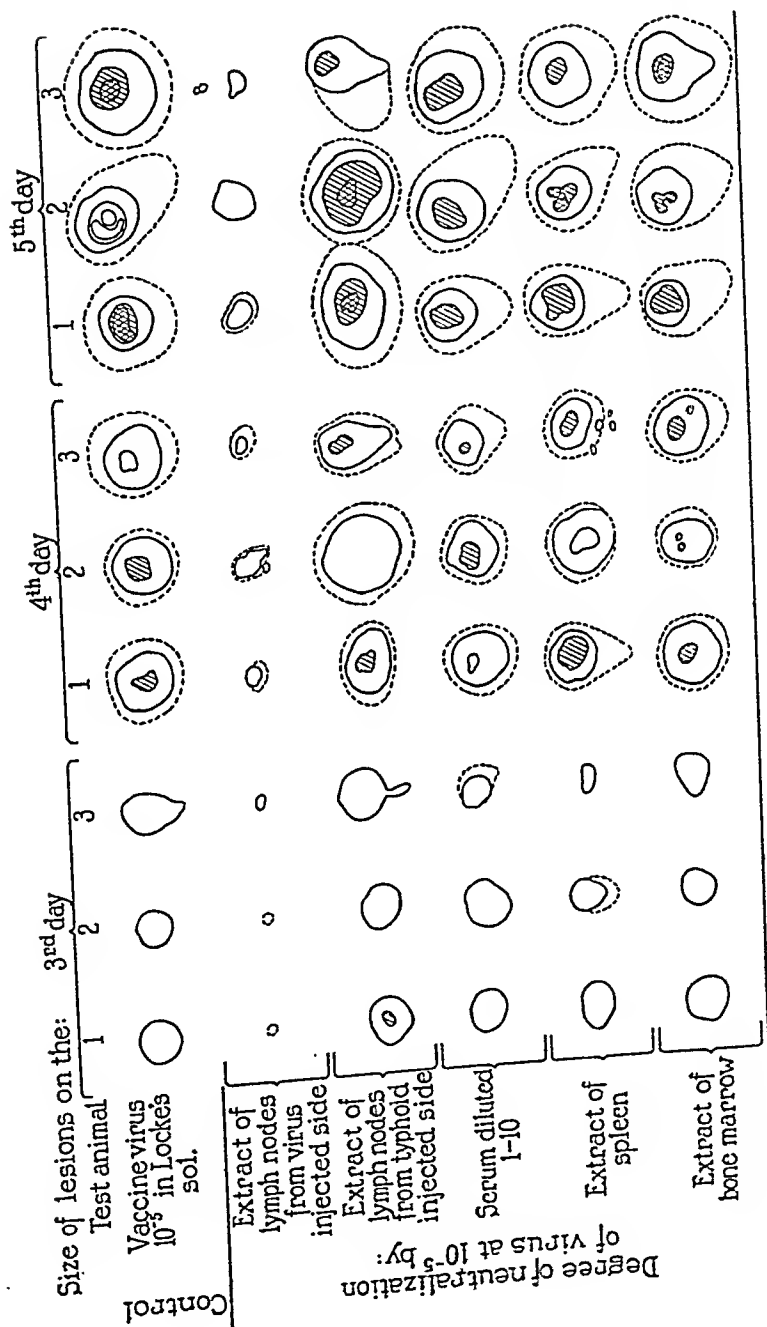
TEXT-FIG. 7—*Continued on Next Page*

Lesions produced by extracts of lymph nodes from virus injected side

No lesions formed on 3rd or 4th day

TEXT-FIG. 7—*Concluded*

TEXT-FIG. 7. Neutralization tests with tissue extracts and serum. Material procured after 6 days.

			Control		Vaccine virus 10^{-9} in Locke's sol.

Extract of lymph nodes from virus injected side

Extract of lymph nodes from typhoid injected side

Serum diluted 1-10

Extract of spleen

Extract of bone marrow

Dilutions 10^{-1} 10^{-2}

Degree of neutralization of virus at 10^{-6} by:

from the virus-injected side, but not by serum similarly diluted or by lymph node material from the uninjected side or by the spleen or bone marrow extracts.

The findings after 5 days yielded still more instructive results. 6 rabbits received virus inoculations in one ear as already described, following which the injected ear was amputated in 3 cases while in the others the injected ear was left intact. All were sacrificed on the 5th day. These experiments were done at different times, and in each necessarily a different frozen specimen of testicular material from the same general batch was used as a source of vaccine virus. Of the 6 animals only one with ear intact and one with the ear amputated received injections of typhoid bacterin into the ear not inoculated with virus. In these instances the nodes were equally enlarged at autopsy, edematous and hemorrhagic. In the other 4, only the cervical lymph nodes of the injected side were inflamed and 6 to 8 times heavier than their companion nodes. The latter seemed in every way normal.

In all 6 experiments the cervical nodes on the virus-injected side showed the presence of antiviral principle as evidenced by some neutralization of the virus on test. In 2 of the 6 instances the extract completely neutralized vaccine virus at a dilution of 10^{-7} and did so largely at 10^{-6}, while the serum in corresponding dilutions showed far less antiviral power, when any, and the other extracts none whatever. The extracts of the cervical lymph nodes of the other 4 animals manifested less antiviral activity and their diluted serum had none at all. In one of these instances the spleen extract neutralized the virus slightly. By titration tests virus was found only in the extracts of the nodes of the virus-injected side. The findings were so similar to those already described that no charts need be given.

3 days after inoculation of virus in one ear an antiviral principle could be detected in the extracts of the cervical lymph nodes of the virus-injected side.

Five experiments of the sort were carried out in the usual way with amputation of the injected ears in three. Typhoid bacterin was injected in the control ears in 2 instances. In these cases the cervical lymph nodes of both sides proved to be greatly and about equally enlarged and hemorrhagic. The findings differed not at all from those in instances in which the bacterin had not been introduced.

Tests showed the presence of virus only in the lymph nodes of the virus-injected side, and the same extract in which it was demonstrated had slight neutralizing power. This was shown by a faint and irregular inhibition of the vaccinia virus in neutralization tests which were made in the usual way. There was no neutralization by the serum and none by extracts of cervical lymph nodes from the uninjected side or by bone marrow or spleen. The tracings of the neutralization tests from this experiment are not shown. The neutralization of virus by the extract of lymph nodes from the virus-injected side was less striking than that of similar node extracts removed on the 4th day after injection of virus in the ear, as described in a following experiment and traced in Text-figs. 8 and 9.

The neutralization tests showed the presence of antiviral principle spread widely through the body of rabbits injected intradermally in

one ear with virus 11 or 15 days before. Extracts of the serum and of various tissues obtained at shorter intervals of time following injection of virus in the ear showed less and less of the neutralizing principle. 1 week after such an injection whole serum, when mixed with vaccine virus suspension, neutralized the virus more than did a 10 per cent extract of the lymph nodes of the virus-injected side, but the latter showed more neutralizing power than the serum when similarly diluted. At an interval of 5 and 6 days after injecting virus in one ear the lymph node extract of the virus-injected side showed more neutralizing power than similarly diluted serum. Even after an interval of 3 days, the lymph node extract showed some neutralizing power and the diluted serum none. An experiment to be described below shows further that after an interval of 4 days the lymph node extracts from the virus-injected side showed more neutralizing power than whole serum.

In these experiments virus could be demonstrated by titration tests in the extracts of lymph nodes from the virus-injected side at the same time that antiviral principle could be demonstrated in the same extracts by the neutralization tests. This finding was obtained in every experiment in which these extracts were made 3 to 7 days after injecting virus in the ear.

Is the Antiviral Principle Developed within Lymph Nodes or Merely Concentrated There?

The lack of neutralizing power of the extracts of the inflamed nodes draining the ear injected with typhoid bacterin would seem to exclude the possibility that antiviral principle, formed elsewhere in the body and circulating in the blood, had collected or been concentrated in the nodes of the virus-injected side. There remained another possible source of the antiviral principle other than the lymph nodes themselves, namely the tissues of the injected ears. The possible formation of bacterial antibodies in the skin has been stressed by Fernbach and Hässler (10), by Cannon and Sullivan (11), Cannon and Pacheco (12) and others. An antiviral principle, formed locally in the ear tissue, might have drained directly by the lymphatics to the lymph nodes, and there accumulated. For virus often remains present for some time in the stump of an ear, injected with virus at its

tip and amputated a few hours later, as shown by the fact that about one-third of our animals so treated developed a definite vaccinia lesion in the ear stump. In about half our experiments the ears injected with relatively small amounts of virus were left intact, with the result that this could drain freely to the cervical nodes. For these reasons an antiviral principle was sought in the ear tissues.

Two rabbits were injected in the usual manner with virus and typhoid bacterin, after which the ears of one were amputated. 4 days later serum and tissue extracts were procured from the tissues of both animals as usual, and in addition ear tissue from the intact ears or stumps on both sides extracted. To obtain the latter the skin and all subcutaneous soft-tissue above the cartilage on the upper side of the ear was removed, from its tip to a point three-quarters of the way to the base of the ear. This included all inflamed tissue, the vaccinia lesion on one side, and the area inflamed by the typhoid vaccine on the other side. In the instance in which amputation of the ear had been done, all soft tissue above the cartilage was removed from the tip of the stump to the base of the ear. The material thus obtained from the etherized, living animal or from one just killed, held practically no blood, for the latter was squeezed out when skin and subcutaneous tissue were stripped from the cartilage. The material was ground with sand and 10 per cent extracts made with Tyrode's solution, as with the other tissues removed. Titration and neutralization tests were carried out as usual with all the extracts and the blood serum. The lymph node extract from the virus-injected side markedly neutralized the virus, that of the typhoid side did not. The ear tissue of the typhoid-injected side yielded no evidence of the presence of virus or of a neutralizing principle, whereas that of the virus-injected side, when injected into the test animals at dilutions of 10^{-5}, 10^{-6} and 10^{-7}, gave rise to large lesions, and when mixed with virus, as in neutralization tests, caused even greater ones.

In the foregoing experiment the procedure employed failed to rule out the possibility that the action of an antiviral principle formed locally in the virus-injected ear had been masked by the presence of much virus. To overcome this difficulty we sought for an antiviral principle in the extract of the tissue of the virus-injected ear by a method known to separate it from virus when both are present together. We resorted to filtration of the greater part of each of the ear and lymph node extracts through Seitz pads. The filtrates of ear and cervical node extracts thus obtained and the extracts themselves were titrated on normal animals to see whether the virus had actually been filtered out, and in addition both extracts and filtrates

were used for neutralization tests. A description of the experiments follows.

Four normal brown-gray rabbits were injected in the left ears with vaccine virus suspension in the manner already described, 2 receiving 0.3 to 0.4 cc. of the virus suspension, the others 0.1 cc. In the right ears typhoid bacterin was injected. 3 hours later the left ears of the 2 receiving the larger dose were amputated under ether. After 4 days the animals with ears intact showed small vaccinia lesions at the injection site. Of the 2 with amputated ears one showed a single small lesion in the ear stump. Serum was procured and the usual tissue extracts made, with, in addition, an extract of the tissue of the ears as in the preceding experiment. It will suffice to give the results in one experiment, in which materials were procured from an animal with the virus-injected ear intact, the ear likely to harbor antiviral principle in its tissue. The other 3 experiments yielded similar results.

Two 10 per cent extracts of ear tissue, one including vaccinia lesions, the other inflamed tissue of the typhoid-injected ear, and the extracts of the cervical lymph nodes of each side as well were filtered through Seitz filters. Enough of each extract was saved so that titration and neutralization tests could be performed with both the unfiltered and filtered material.

The neutralization tests were carried out upon 6 normal rabbits in the usual way. For comparative purposes injections were also made of ordinary virus suspension at the same dilutions, 10^{-5} and 10^{-6}. The employment of so many test animals in this experiment avoided the injection of excessive amounts of vaccine virus in any one individual, which might have led to secondary activation of lesions produced by the neutralization mixtures. The experiment carried out in this way contained its own control as concerned the efficacy of the filtration method to separate vaccine virus from an antiviral principle when both were present in the ear extract. For both were known to be present in the lymph node extract and they were successfully separated.

Titration tests on some additional animals disclosed the presence of virus only in the extracts of the cervical nodes and of the ear tissue on the virus-injected side. It could not be found in the filtrates of these extracts nor in any of the other extracts or filtrates.

Text-figs. 8 and 9 show the results of the neutralization tests made with vaccine virus diluted 10^{-5} and 10^{-6} respectively. The Seitz filtration had effectively held back virus and allowed the passage of the antiviral principle in the extract of cervical lymph node of the virus-injected side; for this filtrate, which on titration test yielded no vaccinia lesions, markedly neutralized vaccine virus. Indeed the filtrate showed greater neutralizing power than the unfiltered extract, a fact explainable by the finding of some free virus in the
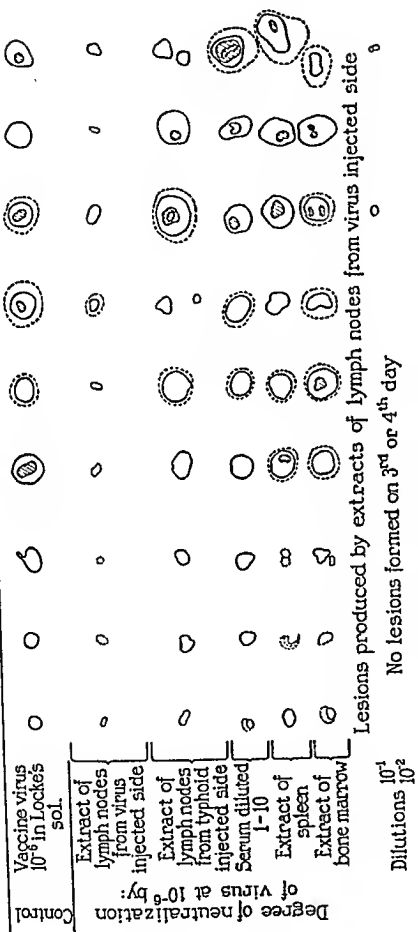
TEXT-FIG. 8. Neutralization tests with tissue extracts, filtrates and serum. Material procured after 4 days.
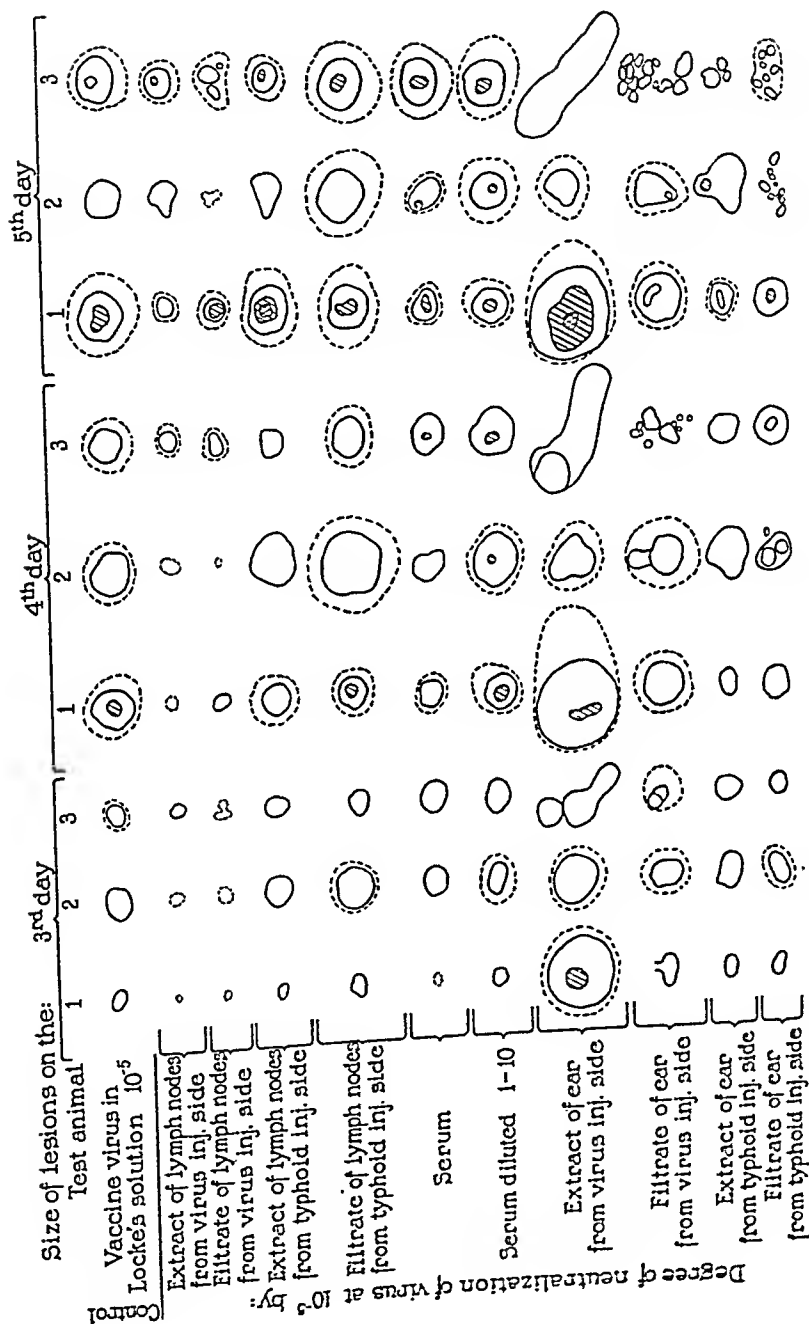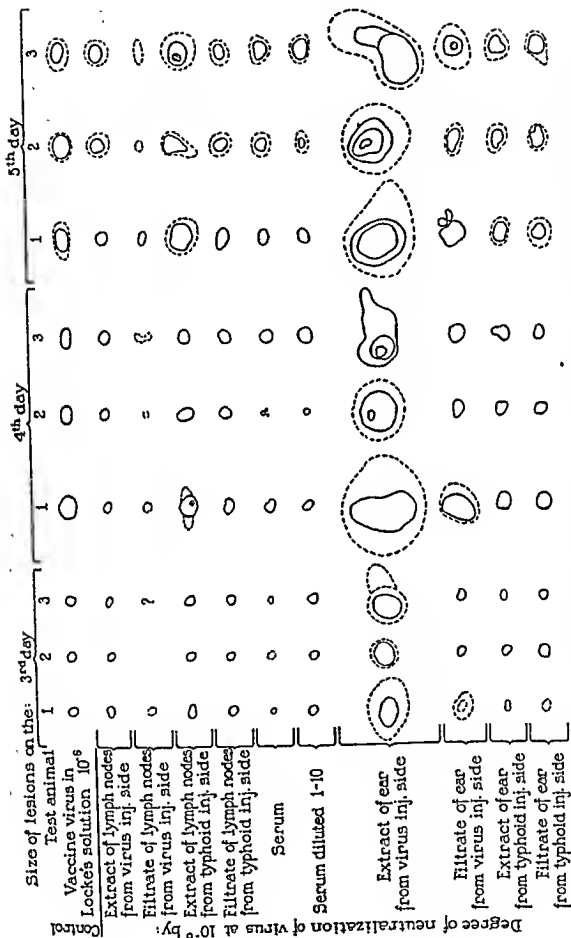
TEXT-FIG. 9. Neutralization tests with tissue extracts, filtrates and serum. Material procured after 4 days.

unfiltered extract, and this when added to that used in the neutralization test gave rise to slightly larger lesions.

In contrast to such findings the filtrate of the extract of the virus-injected ear itself effected no neutralization of virus. It follows that there was no evidence of an antiviral principle in the ear tissue which might have been transmitted to the cervical lymph nodes. The extract of this ear contained much virus as shown by the titration tests, and when mixed with vaccine virus in neutralization tests yielded greater lesions than did the latter alone. Neither the node nor ear extracts or filtrates from the typhoid-injected side caused any neutralization of virus.

The results of tests with whole serum and with serum diluted 1 in 10 have also been charted. Neither show as much neutralization of virus as that caused by the extract of lymph node from the virus-injected side, the former showing some influence, the latter somewhat less. The finding is striking when compared with those described in the 7 day experiment of Text-figs. 5 and 6.

DISCUSSION

The data prove that the regional lymph nodes elaborate an antiviral principle when virus is brought to them by way of the lymphatics from the injected ear, and that this is demonstrable within 4 days after the virus inoculation. The experiments indicate that it could probably be demonstrated even after a shorter time, following an injection of virus in the ear, were better testing methods utilized, as for example if protective substances and virus were separated by filtration or by electrophoresis (13). The antiviral principle was present in greater concentration in the extract of the lymph nodes of the virus-injected side than in the undiluted blood serum procured at the same time, and in far greater concentration than in the serum diluted equally with the node extract. The possibility would seem to have been excluded that an antiviral principle developing in the injected ear or elsewhere in the body accumulated in the lymph nodes, thus accounting for the findings. The control injections of typhoid vaccine inflamed both the injected ear and the nodes draining it, but no antiviral principle was ever found in either until long after their appearance in the nodes of the virus-injected side.

It may well be that under the conditions of the experiments, in which virus reached the nodes on the lymph stream in small amounts and generalized vaccinia did not occur, the lymph nodes played a major rôle in producing the neutralizing principle found in the serum. Certainly the antiviral principle developed first in the nodes. The circumstances were much like those of natural infection through cuts or abrasions in the skin or mucous membranes, with retention of the infecting agent by the draining nodes, and they were so like those of artificial vaccination that there is no need to stress this point. The immunity following clinical vaccination may well be of lymph node origin in great part.

The relative amounts of antiviral principle present in the various organs or body fluids change much with the lapsed time after virus infection. For example the extracts and filtrates from cervical lymph nodes of the virus-injected side, procured 4 days after injection and diluted 10-fold, inhibited the activity of vaccine virus slightly more than did whole serum. Serum diluted 10-fold had little or no effect upon the activity of virus. After 7 days serum and node extract equally diluted show almost equal inhibitory powers, with the serum slightly the weaker. Whole serum showed definitely more neutralizing ability than did gland extract. One may infer that some at least of the antiviral principle now present in the serum was derived from the nodes. Whether it all came from them has still to be determined. The results in any individual case may depend largely upon the portal of entry of the virus, upon the quantity entering and upon whether it spreads rapidly or is retained by one tissue or organ.

We have not sought to ascertain the earliest moment at which the antiviral principle appears in the lymph nodes. Much must depend upon the amount of virus reaching these organs.

A word should be said concerning the methods employed. In our earlier experiments (4) on the formation of agglutinins in lymph nodes of mice, killed cultures or organisms were intradermally injected into ears which were later amputated. The amputation was done to exclude a seepage of antibodies from the dilated blood vessels of the inflamed ear to the interstitial tissue from which they might be drained by lymphatics to the lymph node. The procedure also prevented the possibility of drainage to the nodes of antibodies which might later

have been formed locally. The bacterin primarily introduced was entirely removed save for an insignificant amount present in lymph capillaries cut across at the time of amputation; and antibodies were not found in the tissue of intact, inoculated ears. The employment of active virus introduced a special difficulty. Amputation of the ear largely prevented seepage of antiviral principle into the inflamed tissue of the ear and thence to the nodes, but it did not prevent an increase of virus in the ear stump and the not infrequent occurrence of vaccinia lesions there. The presence of virus might well have acted to stimulate the local development of antiviral properties, were this possible, and the possibility would have existed even if intra-lymphatic injections had been made. It would have been ideal for the purposes of our work if one had been able to instil a known quantity of virus into the afferent channel of a lymph node without the infection of other tissues. But even under such circumstances virus would doubtless have passed from the lymphatic channel to proliferate in the tissues.

SUMMARY

An antiviral principle is elaborated within the regional lymph nodes draining skin into which vaccinia is injected. The immunity conferred by clinical Jennerian vaccination may be largely of lymph node origin.

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MECHANISM OF THE LYSIS OF PNEUMOCOCCI BY FREEZING AND THAWING, BILE, AND OTHER AGENTS

By RENÉ J. DUBOS, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

Bile has the property of killing rapidly, and lysing completely, the cells of most strains of pneumococci (1). Not only has this reaction been used for the differentiation of the Pneumococcus and Streptococcus groups, but the possibility of obtaining solutions of pneumococci has been of some help in physiological and immunological studies of these organisms. The solubility of pneumococci in bile (or better in bile salts and unsaturated fatty acids) is commonly ascribed to the peptizing action of these agents and to their activity as depressors of surface tension. Neufeld observed that in addition to many proteins, lipids, and sterols, the protoplasm of "naked" bacteria (*nacktes Bakterien protoplasma*) is readily soluble in bile; he explained the difference in the behavior of streptococci (insoluble in bile) and pneumococci (soluble in bile) as due to a more porous structure of the cell membrane in the latter group of microorganisms (1). Lord and Nye (2), however, and later Atkin (3), voiced the opinion that bile solubility is only an activation of the normal autolytic process, and that the lysis itself is caused by the cellular enzymes. This point of view was accepted by Mair (4).

The breaking up of pneumococci can also be achieved by repeated freezing and thawing of the cells; in this instance, the stress due to the formation of intracellular ice crystals has been regarded as causing the disruption of the cells. It is a matter of importance, both for physiological and immunological studies, whether the techniques used for breaking up pneumococci involve only a change in colloidal state (peptizing action of bile salts and fatty acids, mechanical disruption caused by ice crystals), or whether they bring about more profound alterations of the components of the cellular structure.

It will be shown in the present paper that pneumococcus cells can be broken up by bile, by freezing and thawing, and by other techniques to be described, only when certain cellular enzymes are present in an active form, and when conditions are favorable for enzymatic action. It is likely, therefore, that when pneumococci are put in solution by these techniques, alterations take place which affect the chemical integrity of the cellular constituents.

EXPERIMENTAL

Methods.—Pneumococci were grown in a peptone-beef heart infusion broth. The cells were collected by centrifugalization from 6 hour old cultures.

Whole ox bile, filtered and autoclaved, was used in varying amounts as described in the experimental part; in some instances, a neutral solution of sodium desoxycholate was employed instead of bile.

The process of freezing was carried out by immersing the cell suspension in an alcohol-carbon dioxide mixture at a temperature of about $-50°C$.

Conditions Which Determine the Bile Solubility of Dead Pneumococci.—Whereas living pneumococci are readily soluble in bile, it is well known that suspensions of these organisms killed by heat, or by the use of formalin, are entirely insoluble in this reagent. The following experiments show, however, that it is possible to kill pneumococci under such conditions that the cells remain soluble in bile.

Experiment 1.—The cells collected from 250 cc. of broth culture of Pneumococcus Type I were resuspended in 25 cc. of M/20 phosphate buffer pH 7.0; this cell suspension was distributed in 3 cc. amounts into eight tubes; iodine was then added in the concentrations described in Table I. The cell suspensions were incubated for 2 hours at 37°C., and the presence of living cells at the end of this time tested by streaking the preparations on blood agar plates. 1 hour later, the cells in each case were washed free of iodine, and resuspended in 3 cc. of M/20 phosphate buffer pH 7.0. Four of the preparations were then treated with 0.5 cc. of bile (tubes 5, 6, 7, 8), and the other four kept as controls (tubes 1, 2, 3, and 4). The eight preparations were incubated at 37°C. The results presented in Table I describe the effect of iodine treatment on the viability of the cells and the microscopic appearance (Gram stain) after 1 hour and 48 hours incubation.

The results of Experiment 1 show that the pneumococci were rapidly killed by the smallest amount of iodine used; when these dead cocci, and even the cocci killed with twice this amount of iodine, were treated with 0.5 cc. of bile, they were found to be readily and completely

soluble. The cells killed with a large excess of iodine, however, had
lost their solubility in bile. It is likely that, in this instance, the
highest concentration of iodine was not only lethal but destroyed the
autolytic enzymes, whereas the latter remained active with the smaller
amount of iodine even though the cells were no longer viable. This is
indicated by the fact that on prolonged incubation (48 hours) the cells
in tubes 2 and 3 underwent spontaneous autolysis; whereas those in
tube 3 were fixed and remained Gram-positive.

TABLE I

Relations between the Viability, Bile Solubility, and Autolysis of Pneumococci Treated with Iodine

Tube	Amount of M/10 Iodine solution added	Growth on blood agar plates	Bile	Microscopic appearance after incubation at 37°C.	
				1 hr.	48 hrs.
	cc.		*cc.*		
1	0	+++++	0	Gram-positive cocci	Gram-negative detritus
2	0.015	−	0	" "	" "
3	0.03	−	0	" "	" "
4	0.1	−	0	" "	Gram-positive cocci
5	0	+++++	0.5	Gram-negative detritus	Gram-negative detritus
6	0.015	−	0.5	" "	" "
7	0.03	−	0.5	" "	" "
8	0.1	−	0.5	Gram-positive cocci	Gram-positive cocci

(columns for "2 hrs. incubation at 37°C." span tubes 1–8)

++++ indicates abundant growth.

− indicates no growth.

The conclusion that it is possible to kill pneumococci without
destroying their autolytic enzymes and their solubility in bile is further
illustrated by another technique in Experiment 2.

Experiment 2.—A broth culture of Pneumococcus Type I was made acid to
pH 4.4 by the careful addition of glacial acetic acid. No living organism could be
recovered from the acidified culture (pH 4.4) kept for 2 hours at room temperature.

The acid culture was held for 1 month under these conditions. After this time
the cells, which were still well shaped and Gram-positive, were centrifugalized and
resuspended in 12 cc. physiological saline; half of this suspension (6.0 cc.) was
heated in a boiling water bath for 10 minutes, and the other half kept unheated.
4.0 cc. of each of the heated and unheated cell suspensions was neutralized by the
addition of alkaline phosphate and the rest (2.0 cc. of each) maintained at an acid

reaction. The materials were finally distributed in 2.0 cc. amounts into test tubes, some of which received in addition 0.5 cc. of bile (Table II). The bacterial suspensions were incubated at 37°C. and stained by the Gram technique after 20 minutes and 48 hours incubation.

The results of Experiment 2 demonstrate that pneumococci killed with acetic acid at pH 4.4 were still Gram-positive and retained their morphological integrity after the acidified culture had stood for 1 month at room temperature. When the same cocci were resuspended in a neutral solution, they proved to be soluble in bile and underwent spontaneous autolysis, indicating that in spite of rapid cell death the autolytic enzymes had not been destroyed after prolonged exposure to

TABLE II

The Effect of Heating upon the Bile Solubility and Autolysis of Pneumococci Killed with Acetic Acid

Cocci killed with acetic acid	pH of cell suspension	Bile	Microscopic appearance after incubation at 37°C.	
			20 min.	48 hrs.
		cc.		
Unheated	4.4	0	Gram-positive cocci	Gram-positive cocci
"	7.0	0	" "	Gram-negative "
"	7.0	0.5	Gram-negative detritus	" detritus
Heated	4.4	0	Gram-positive cocci	Gram-positive cocci
"	7.0	0	" "	" "
"	7.0	0.5	" "	" "

pH 4.4. If on the other hand, the enzymes in these same cells were destroyed by heating to the boiling point previous to neutralization, the cocci lost at the same time their ability to undergo autolysis and their bile solubility.

Similar results were obtained when the cells were killed with citric acid instead of acetic acid. When, however, hydrochloric acid or phosphoric acid was used, the results were very unsatisfactory. This is probably due to the greater difficulty with which mineral acids penetrate the cells in contrast to organic acids, and the consequent difficulty in establishing rapidly an intracellular acidity sufficient to inactivate the enzymes, but not great enough to destroy them irreversibly.

The Effect of Reaction upon the Solubility of Pneumococci in Bile.—
Lord and Nye (2), observed a certain parallelism between the rates of
autolysis and of bile lysis of pneumococcus suspensions at different
pH and suggested that bile lysis may be only a modified form of the
normal autolytic process. Since the bile acids and unsaturated fatty
acids are insoluble under acid conditions, the rates of autolysis and bile
lysis were compared at different alkaline reactions.

Experiment 3.—The cells collected from 150 cc. of culture of Pneumococcus
Type III were divided into six equal portions each of which was resuspended in
4 cc. amounts of M/10 phospbate buffer at pH 7.5 and 8.0 and of M/20 NaOH in
duplicate series. To one series 0.5 cc. of bile was added; the other series without

TABLE III

*The Effect of Alkaline Reactions upon the Rates of Autolysis and Bile Lysis of
Pneumococci*

Bacteria in 4.0 cc. of	Bile	Microscopic appearance after incubation at 37°C.			
		20 min.	1 day	3 days	7 days
	cc.				
Buffer pH 7.5	0	Gram-positive	Gram-negative	—	—
Buffer pH 8.0	0	"	Gram-positive	Gram-negative	—
M/20 NaOH	0	"	"	Gram-positive	Gram-positive
Buffer pH 7.5	0.5	Gram-negative	—	—	—
Buffer pH 8.0	0.5	"	—	—	—
M/20 NaOH	0.5	Gram-positive	Gram-positive	Gram-positive	Gram-positive

bile was kept as control. The preparations were incubated at 37°C., and at
different intervals of time, stained by the Gram technique. Tbe results are
presented in Table III.

The results of Experiment 3 indicate that the process of autolysis
is slower at pH 8.0 than at pH 7.5 and completely inhibited in M/20
NaOH. Bile lysis proceeds so rapidly that no difference in rate could
be detected between pH 7.5 and pH 8.0; in M/20 NaOH, the pneu-
mococci appeared completely resistant to the action of bile.

The Effect of Temperature upon the Bile Lysis of Pneumococci. Experiment 4.—
The cells collected from 150 cc. of culture of Pneumococcus Type III were resus-
pended in 32 cc. of M/10 pbosphate buffer pH 7.4 and distributed equally into
eight tubes. Sodium desoxycholate was added in two different amounts and tbe

preparations incubated simultaneously at four different temperatures (22°, 37°, 50°, and 60°C.). The course of lysis was followed by comparing relative differences in the turbidity of the cell suspensions (Table IV).

The results of Experiment IV indicate that the rate of lysis of pneumococci by sodium desoxycholate increases with temperature up to 50°C. and with increasing concentration of the bile salt. At 60°C., some lysis was observed to take place immediately, but the bacterial suspension never cleared completely; this may be traced to the rapid inactivation of the autolytic enzymes at high temperature.

TABLE IV

The Effect of Temperature on Lysis of Pneumococci by Different Concentrations of Sodium Desoxycholate

1 per cent solution of sodium desoxycholate	Temperature of incubation	Minimum time required for complete lysis*
cc.	*°C.*	
0.02	22	Not complete in 3 hrs.
0.02	37	3 hrs.
0.02	50	40 min.
0.02	60	No lysis in 3 hrs.
0.2	22	20 min.
0.2	37	15 min.
0.2	50	5 min.
0.2	60	Not complete in 3 hrs.

* The degree of lysis was estimated in terms of the turbidity of the cell suspension.

Experiment 5.—The cells collected from 150 cc. of broth culture of Pneumococcus Type I were resuspended, half in 10.0 cc. of M/20 borate buffer pH 8.0, and half in 10.0 cc. M/20 borate buffer pH 8.6. The two suspensions were chilled in an ice bath, and to each was added 1.0 cc. of chilled 10 per cent sodium desoxycholate. The progress of lysis was recorded in terms of clearing of the cell suspensions. The preparation at pH 8.0 was appreciably clearer after 1 hour in the ice bath, and completely lysed after 3 hours; the preparation at pH 8.6 was still somewhat cloudy at that time.

As soon as the bile salt was added to the bacterial suspension, small samples of each preparation were removed and incubated at 37°C. The preparation at pH 8.0 cleared instantaneously, whereas the more alkaline suspension remained somewhat cloudy for almost 2 hours. .

The Effect of Freezing upon Pneumococci.—It was shown in Experiments 1 and 2, that pneumococci killed under conditions such that the autolytic enzymes were not destroyed (moderate amount of iodine, or acetic acid at pH 4.4) remained soluble in bile and capable of undergoing autolysis. These two properties were lost, however, when the enzymes were destroyed by an excess of iodine or by heating to the boiling point. Interestingly enough the breaking up of pneumococci by repeated freezing and thawing seems to be governed by the same laws, and without describing the experimental details, one may state that dead pneumococci remain susceptible to disruption by the freezing and thawing technique as long as the autolytic enzymes of the cells remain potentially active. If then, the cellular breakdown which follows freezing and thawing is in some way related to the autolytic process, one must assume that in cells which have been exposed to these physical conditions, the autolytic enzymes can function more rapidly than they do in the normal cell. The effect of freezing upon pneumococci is illustrated in the following experiment.

Experiment 6.—The cells collected from 1000 cc. of culture of Pneumococcus Type III were resuspended in 30.0 cc. of M/20 phosphate buffer pH 7.0. This suspension of living cells was distributed in 3.0 cc. amounts into four pyrex tubes, two of which (tubes 1 and 2) were kept for 3 hours in a bath of alcohol and carbonic ice (temperature about −50°C.) and the other two (tubes 3 and 4) kept at 0°C. for the same length of time. At the end of this period, two of the preparations (tubes 1 and 3) were treated with 10.0 cc. of M/2 NaOH and allowed to incubate at 45°C. in the alkaline medium. The materials in tubes 2 and 4 were also incubated at 45°C. for 30 minutes and only then treated with 10.0 cc. of M/2 NaOH. At the end of the incubation period, the preparations were stained by the Gram technique (Table V).

It appears from the results of Experiment 6 that the cells which have been frozen at −50°C. undergo lysis very rapidly when the temperature is raised. The incubation time was in reality much shorter than 30 minutes since the frozen material had to be thawed before reaching 45°C.; most of the cells which had been kept at 0°C. remained intact during the incubation at 45°C. Since no lysis occurred when the frozen cells were incubated in the alkaline solution, it is likely that the disruption of the cells in tube 2 was due to the

activity of the autolytic enzymes.   The essential rôle of these enzymes in the lysis which follows freezing is again demonstrated in the following experiment.

Experiment 7.—The same cell suspension described in Experiment 6 was distributed in 3.0 cc. amounts into two pyrex tubes.   The two preparations were frozen and thawed six times, but the temperature of the material was never allowed to go above 0°C. even in the thawed condition.   This was achieved by freezing at very low temperature (−50°C.) and thawing in the presence of 5.0 cc. saturated NaCl (to lower the melting point of the frozen suspension of pneumococci).   At the end of the sixth thawing, one of the preparations was treated with 1.0 cc. of N/10 iodine to stop any enzymatic action, then incubated for 30 minutes

TABLE V

The Effect of Freezing upon the Autolysis of Pneumococci

Tube	Cells kept for 3 hrs. at	Treatment	Microscopic appearance
	°C.		
1	−50	10.0 cc. N/2 NaOH added, then incubated for 30 min. at 45°C.	Gram-positive cocci
2	−50	Incubated 30 min. at 45°C., then 10.0 cc. N/2 NaOH added	Gram-negative detritus
3	0	10.0 cc. N/2 NaOH, then incubated 30 min. at 45°C.	Gram-positive cocci
4	0	Incubated 30 min. at 0°C., then 10.0 cc. N/2 NaOH added	Some Gram-negative cells. Large number of Gram-positive cocci

at 45°C.; the other preparation was incubated at the same temperature for the same length of time, and the iodine solution added only at the end of the incubation period.   Films stained by the Gram technique revealed only Gram-positive cocci in the *former* preparation and only Gram-negative detritus in the latter.

This experiment demonstrates that mere freezing and thawing, even though repeated six times, does not bring about disruption of the cells of living pneumococci, if, immediately following the treatment, iodine is added in concentration sufficient to inhibit enzyme action; however, the cells which have been frozen and thawed undergo lysis as soon as conditions become favorable for enzymatic action.

The "Flash" Lysis of Pneumococci Desiccated by Cold Acetone.—If a suspension of live pneumococci (R or S forms) is precipitated with ten

volumes of cold acetone, the acetone immediately removed, the material washed with cold acetone and dried *in vacuo*, one obtains a powder which illustrates in a startling manner the tremendous activity of the autolytic system of the pneumococcus cell.

A small nmount of desiccated cells, resuspended in saline or neutral buffer solution, and immediately smeared on a glass slide, reveals under the microscope only Gram-negative detritus. The cells appear to have been disrupted by the acetone treatment. If, however, the bacterial powder is resuspended in a medium capable of inhibiting or destroying the cellular enzymes (boiling water, dilute acid, dilute ammoniacal solution, iodine solution, formaldehyde, etc.) the material is found to consist of Gram-positive cocci. Once more, therefore, the change in physical state associated with rapid desiccation with acetone at low temperature has so modified the cells that the normal processes of autolysis are greatly accelerated as soon as the conditions favorable for enzymatic action are restored.

Toluol, ether, tricresol, many dilute nntiseptics constitute other examples of agents which activate the autolysis of pneumococci, but in all instances, the cells retain their characteristic morphology and lysis fails to take place when the cellular enzymes are destroyed or prevented from exerting their lytic action. Low temperature, dilute acetic acid, dilute ammonia, other inhibitors of enzyme action, suffice to render ineffective all the techniques so far devised to cause the dissolution of living pneumococci.

DISCUSSION

It has been repeatedly shown in the preceding experiments that, to be soluble in bile, or liable to disruption by freezing and thawing, the pneumococci, living or dead, must still possess their autolytic enzymes in a potentially active form. Since enzymes are probably of protein nature, and probably active only in the native state, the previous observation might be interpreted as evidence that with denaturation of the cell proteins (and therefore inactivation of the enzymes) there is at the same time a loss of bile solubility. This explanation, however, does not account for all the facts. If pneumococci are to be broken up by bile treatment, or by freezing and thawing, it is not sufficient that the cellular enzymes be potentially active, it is also

essential that the conditions be favorable for enzymatic action. It appears, therefore, that dissolution of the cells involves the action of certain though not necessarily all of the autolytic enzymes. Does it mean that bile lysis, for instance, is identical with normal autolysis? This appears not to be the case. In fact, Goebel and Avery have shown that pneumococci can be put in solution by large concentrations of desoxycholate without any appreciable proteolysis; the bile salt acts as an inhibitor of the proteolytic enzymes (5). Bile lysis, as well as freezing and thawing, may involve as a necessary step only one, or a few, of the many stages of the autolytic complex.

In addition to pneumococci, several protozoan and bacterial species are also soluble in bile. It is likely, however, that the mechanism of bile lysis is not the same in all instances. We have found, for instance, that Pfeiffer bacilli (*Hemophilus influenzae*) remain entirely soluble in bile after they have been killed by heat, and also in the presence of $N/5$ NaOH. This is in marked contrast to the conditions which obtain in the case of pneumococci and corresponds to the thesis upheld by Neufeld (1) and described in the introduction of the present paper. Pneumococci are Gram-positive organisms, Pfeiffer bacilli Gram-negative; one may wonder whether this difference in staining reaction is not associated with the difference in behavior of the two bacterial species with reference to the action of bile. It is perhaps worth considering that the substrate which has to be attacked by the autolytic enzymes to render pneumococci soluble in bile is the Gram-positive structure which has been described in a previous paper (6).

If the effect of bile, or of freezing and thawing is an indirect one, through the agency of some autolytic enzyme, what is the nature of their direct effect upon the cell? We have seen that when live pneumococci are frozen at very low temperature, or desiccated by acetone, the cells are so modified that the autolytic enzymes, which are held in abeyance in the normal cell, begin to function with great speed as soon as conditions become favorable for enzymatic action. Why this increased activity? Are the enzymes modified? Or the substrates upon which they function? It is also possible that there exists in the normal cell a certain cellular structure which prevents the enzyme from attacking its specific substrate and that this structure is destroyed by the treatment which causes lysis.

Wollman (7, 8) has already shown that a great many antiseptics have the interesting property of activating the autolysis of different bacterial species when used in low concentrations, whereas higher concentrations of the same agents completely stop the autolytic process. This we have found to be true in the case of Pneumococcus and examples of this dual action will be described in subsequent studies. But it may be proper to point out at this time, that the hypothesis of the existence of a protective structure between the autolytic enzyme and its specific substrate, may help to explain certain discordant results. A great variety of agents (antiseptics) would have the property of breaking down this barrier between the autolytic enzyme and its substrate and therefore promote lysis. Some of these same agents, however, when used in high concentration, also inactivate the autolytic enzyme and therefore maintain the gross morphological integrity of the cell. Such is the case with phenol and formaldehyde, for instance.

In view of these observations, lysis of pneumococci may be regarded as the result of an injury inflicted upon the cell by an agent or a procedure which not only kills the cell without destroying the autolytic enzymes, but also maintains conditions favorable for their activity.

SUMMARY

Pneumococci, living or dead, are soluble in bile when: (a) the autolytic enzymes are still present in a potentially active form; (b) conditions are favorable for enzymatic action.

Bile solubility of pneumococci involves as a necessary step one, or a few, of the many stages of the autolytic complex.

These observations hold true for the disruption of pneumococci by freezing and thawing, by previous desiccation with cold acetone, and by dilute solutions of antiseptics.

A possible mechanism is discussed to account for these forms of lysis.

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THE EFFECT OF THE BACTERIOLYTIC ENZYME OF PNEUMOCOCCUS UPON THE ANTIGENICITY OF ENCAPSULATED PNEUMOCOCCI

By RENÉ J. DUBOS, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

Under appropriate conditions, the immunization of rabbits with heat-killed encapsulated pneumococci incites the production of type specific precipitins, directed against the capsular polysaccharides of the strains used as antigens. It is known, however, that in the rabbit the polysaccharides themselves cannot function as antigens, at least not in the form in which they have been extracted in solution from the bacterial cells. The intact heat-killed cell therefore appears to contain an antigen for which the capsular polysaccharide behaves as a hapten. Surprisingly enough, all efforts to obtain this hypothetical capsular polysaccharide antigen in solution have so far failed. Moreover, it has been found that virulent pneumococci disintegrated by autolysis, by bile, or by freezing and thawing fail to incite rabbits to produce the type specific precipitins directed against the capsular polysaccharides, whereas intact cocci function as efficient antigens (1). In a preceding paper it has been demonstrated that the disintegration of pneumococci by bile or by freezing and thawing involves as an essential step, the action of some at least of the enzymes which constitute the autolytic system of the cell (2). It appears likely, therefore, that under the conditions outlined above, the loss of antigenicity suffered by pneumococci during lysis is to be traced to the action of intracellular enzymes upon the capsular polysaccharide antigen. Evidence for this point of view is presented in the following experiments.

EXPERIMENTAL

Cultures.—Virulent pneumococci of Types I, II, and III were grown and preserved in blood broth and passed through mice often enough to maintain a

degree of virulence such that 0.000,000,01 cc. of an 8 hour culture would regularly kill 18 gm. mice within 72 hours. Cultures of R variants were similarly grown in blood broth.

Heat-Killed Cells.—Young pneumococci were resuspended in a small amount of distilled water and rapidly added to a larger volume of distilled water at 75°C.; the temperature was maintained at this level for 20 minutes. This process of "flash" heating was selected because it minimizes the chances of alterations due to enzymatic action (3). The volume of fluid was so arranged that the final concentration of cells in the bacterial suspension was fifteen times greater than in the original culture.

Bacteriolytic Enzyme.—The enzyme was used in the form of a crude autolysate, or of a filtered solution, or of a preparation partially purified by precipitation with flavianic acid. These three different preparations are described in a preceding paper (3).

Methods of Immunization.—The cell suspensions were injected into rabbits intravenously on 6 consecutive days, followed by a free interval of 1 week. Three courses were given, each animal receiving daily a dose containing the cells from 1 cc. of broth culture. The rabbits were bled on the 8th day after the last injection of each course.

Mice were given three intraperitoneal injections, at 3 day intervals, of heat-killed cocci resuspended in 0.5 cc. physiological saline. The immunizing dose is described for each experiment. 6 days after the last immunizing injection, the mice were infected by the intraperitoneal injection of virulent pneumococci as described for each experiment.

Serological Tests.—In all precipitin reactions, 0.5 cc. of immune serum, or 0.2 cc. of the serum diluted to 0.5 cc. volume with salt solution, was added to an equal volume of graded dilutions of the purified capsular polysaccharides. The mixtures were incubated for 2 hours at 37°C.; the final readings were made after keeping the reacting mixtures overnight in the ice box.

The Effect of the Bacteriolytic Enzyme upon the Antigenicity of Heat-Killed Pneumococci in Mice.—It is known that when heat-killed pneumococci are treated with appropriate amounts of pneumococcus autolysates, the cells are disintegrated into Gram-negative detritus (4). The effect of this treatment upon the antigenicity in mice of Type I pneumococci is considered in Experiment 1.

Experiment 1.—A mixture in equal volumes of a suspension of heat-killed pneumococci Type I and of active autolysate prepared from the same strain was incubated for 3 days at 37°C. Marked clearing of the cell suspension occurred and stained films revealed the presence of only Gram-negative detritus.

To serve as control a portion of the same suspension of heat-killed cells was incubated for the same length of time with an equal volume of the same autolysate

which had been inactivated by heating in a boiling water bath for 10 minutes. In this case the heat-killed cells retained their characteristic morphology and staining reactions.

Four groups of sixteen mice each were immunized with these preparations, as indicated in Table I. Some of the mice received a total amount of 0.6 cc. of cell

TABLE I

The Effect of the Bacteriolytic Enzyme upon the Antigenicity in Mice of Type I Pneumococci

Immunizing materials		Infecting organisms				
Heat-killed pneumococci Type I treated with	Total immunizing dose	Infecting dose	Pneumococcus Type I		Pneumococcus Type II	
	cc.	*cc.*				
Active enzyme	0.6	10^{-3}	D60	D60	D18	D18
		10^{-4}	D72	S	D18	D36
		10^{-5}	D96	S	D18	D36
		10^{-6}	D60	S	D36	D48
Active enzyme	0.12	10^{-3}	D60	D60	D18	D18
		10^{-4}	S	S	D18	D18
		10^{-5}	S	S	D36	D36
		10^{-6}	S	S	D36	D36
Heat-inactivated enzyme	0.6	10^{-3}	S	S	D18	D36
		10^{-4}	S	S	D36	D36
		10^{-5}	S	S	D36	D36
		10^{-6}	S	S	D36	D36
Heat-inactivated enzyme	0.12	10^{-3}	S	S	D18	D18
		10^{-4}	S	S	D18	D18
		10^{-5}	S	S	D36	D36
		10^{-6}	S	S	D36	D36
Control mice		10^{-6}	D60		D18	
		10^{-7}	D60		D36	
		10^{-8}	D60		D48	

S, survival of the animal.

D, death of the animal; the numeral indicates the number of hours before death.

suspension (containing the heat-killed cells from 4.5 cc. of the original culture); the others received a total amount of only 0.12 cc. of the same cell suspension. 6 days after the last injection of antigen, the animals were infected with different doses of virulent cultures of Pneumococcus Type I or Type II. The survival or death of the infected animals is indicated in Table I.

It appears from the results presented in Table I that all the mice immunized with the control intact bacterial cells (treated with the heated enzyme) were protected against the maximal infecting dose of Type I Pneumococcus used in the test (0.001 cc.). Immunization with the lysed cocci induced a variable degree of active immunity which, however, was less than that developed in response to the control antigen. In all instances the protection acquired was sharply type specific.

It may seem strange that immunization with the smaller amount of disintegrated pneumococci (0.12 cc.) induced more efficient immunity than did the larger amounts of the same material (0.6 cc.). This is not, however, an unprecedented observation. Schiemann and Casper (5) immunized mice against infection with virulent pneumococci by the injection of preparations of the capsular polysaccharide of the same specific type as that of the organisms used as infective agent; they observed, however, that to be antigenically effective, the polysaccharide had to be used within a limited range of concentration; when too much was injected, no immunity resulted. Similarly, Avery and Goebel (6), following the studies of Enders (7) and of Wadsworth and Brown (8), prepared from Type I pneumococci an acetylated form of the capsular polysaccharide which was antigenic in mice under the conditions described by Schiemann and Casper. There exists, therefore, an analogy between the irregular type of immunization described in Table I and the results of Schiemann and Casper, and of Avery and Goebel by the use of the polysaccharides alone. This analogy suggests that the protection induced in mice by immunization with disintegrated pneumococci may be due to the small amount of free polysaccharide present in the preparation.

The amount of bacteriolytic enzyme used in Experiment 1 was sufficient to cause complete disintegration of the heat-killed pneumococci. However, it has been shown in a previous paper (3) that it is possible to render the bacteria Gram-negative without affecting their characteristic morphology or causing an actual lysis of the cells. When Gram-negative bodies, prepared from Type I pneumococci by the use of small amounts of enzyme, were tested for their ability to stimulate active immunity in mice, results similar to those of Experiment 1 were obtained.

The Effect of the Bacteriolytic Enzyme upon the Antigenicity of Pneu-mococci in Rabbits.—It is known that the chemically purified capsular polysaccharides of Pneumococcus cannot function as antigens in the rabbit. If the active immunity induced in mice (Experiment 1) by the immunization with Type I pneumococci treated with the active bacteriolytic enzyme is due solely to the presence of small amounts of free acetyl polysaccharide, then the same materials should fail to

TABLE II

The Effect of the Bacteriolytic Enzyme upon the Antigenicity in Rabbits of Type I Pneumococcus

Immunizing material. Pneumococci Type I treated with	Rabbit	Precipitin reaction					
		2nd course of immunization			3rd course of immunization		
		Final dilution of SSS I			Final dilution of SSS I		
		1:10,000	1:50,000	1:100,000	1:10,000	1:50,000	1:100,000
Heat-inactivated enzyme	1	+	++	++	+	+++	+++
	2	+	++	++	+	++	+++
	3	+	++	++	+	+++	+++
Active enzyme	4	−	−	−	−	−	−
	5	−	−	−	−	−	−
	6	−	−	−	−	−	−
	7	−	−	−	−	−	−
	8	−	−	−	−	−	−
	9	−	−	−	−	−	−

+ indicates the intensity of the precipitin reaction.
− indicates that no precipitation took place.

incite the production in rabbits of the homologous type specific precipitin. This is established in the following experiment.

Experiment 2.—The immunizing materials were the same preparations used in Experiment 1. Three rabbits were immunized with the control bacteria treated with the heat-inactivated enzyme, while six animals received the cocci treated with the active enzyme. The animals were bled after the second and third course of immunization and their sera were tested for the presence of precipitins for Type I capsular polysaccharide. The results are presented in Table II.

The results of Experiment 2 indicate that the serum of the three rabbits immunized with the control intact bacteria was capable of precipitating the Type I capsular polysaccharide; whereas the serum of the six animals which had received the disintegrated pneumococci failed to react even after the third course of immunization.

A decrease in antigenicity was also observed when the pneumococci were merely rendered Gram-negative, but not disintegrated. This is shown in the following experiment.

Experiment 3.—The heat-killed bacteria collected from 150 cc. of Type I pneumococcus culture were resuspended in 15 cc. of M/20 phosphate buffer pH 6.0, and treated with 1.0 cc. of a solution of bacteriolytic enzyme (prepared from an R strain derived from Type II) which had been purified by precipitation with flavianic acid (3). The cells exposed to the action of the enzyme for 18 hours at 37°C. had become Gram-negative, but had not lost their morphology and there was no clearing of the cell suspension. The Gram-negative cells were centrifugalized, resuspended in the same enzyme solution, incubated, and the same process again repeated. The bacteria were finally resuspended in physiological saline, and the cell suspension was found to have retained the same appearance as after the first digestion.

To serve as controls, heat-killed pneumococci (Type I) were subjected to the same treatment described above (incubation at 37°C. in buffer pH 6.0, repeated washings) but without being exposed to the action of the enzyme; these bacterial cells remained intact and Gram-positive.

The two rabbits immunized with the control intact bacteria developed type specific precipitins for the capsular polysaccharide after the first course of immunization and the antibody titer increased as the immunization was continued. On the other hand, the sera of the three rabbits which had received the Gram-negative cocci still failed to give the precipitin reaction even after the third course of immunization.

Two conclusions can be derived from this experiment. (*a*) An enzyme prepared from an R strain derived from Type II, is capable of inactivating the capsular polysaccharide antigen of Type I pneumococci. (*b*) Virulent pneumococci lose the ability of inciting rabbits to form the type specific carbohydrate antibody when they are rendered Gram-negative, even though the cells retain their characteristic morphology.

In the preceding experiments the bacteria used for immunization had been killed by heat; it will be shown in the following experiment

that the bacteriolytic enzyme is equally effective against cells killed by iodine.

Experiment 4.—The cells collected from 150 cc. broth cultures of Pneumococcus Types I and III were respectively resuspended in 10 cc. N/100 iodine and exposed to this reagent at 37°C. for 24 hours. This amount of iodine was sufficient to cause an irreversible inactivation of the cellular autolytic enzymes, and therefore to fix the cells as Gram-positive cocci (3). The iodinated cells were then washed twice with saline and resuspended in 7.5 cc. saline. Autolysates of Pneumococcus Type I and Type III were prepared as usual.

Aliquot portions of the suspensions of the iodinated cells were then mixed with equal volumes of the autolysates as indicated in Table III, and the mixtures were

TABLE III

The Effect of the Bacteriolytic Enzyme upon the Antigenicity in Rabbits of Iodinated Pneumococci

Immunizing materials		No. of rabbits	Precipitin reaction (final dilution of serum 1:5)	
			Polysaccharide (final dilution 1:100,000)	
Iodinated pneumococci	Active enzyme prepared from pneumococci		SSS I	SSS III
Type I	—	1	+	—
" I	Type I	3	—	—
" I	" III	3	—	—
" III	—	1	—	+
" III	Type I	3	—	—
" III	" III	3	—	—

incubated at 37°C. for 4 days; at the end of this time, the cells-autolysates mixtures revealed only Gram-negative detritus.

Rabbits were immunized by the intravenous route with the suspensions of digested bacteria or with untreated Gram-positive iodinated cocci. The animals were bled after the second course of immunization and their sera tested for the presence of precipitins directed against the Type I and Type III capsular polysaccharides (Table III).

The results of Experiment 4 indicate that the rabbits immunized with the Gram-positive iodinated cocci developed precipitins directed against the capsular polysaccharide derived from the same specific type as that of the cells used as antigen; sera of all the other animals were negative for both polysaccharides. The following conclusions

appear therefore justified. (*a*) The bacteriolytic enzyme inactivates the capsular polysaccharide antigen of iodinated pneumococci. (*b*) This result is obtained with enzymes prepared from heterologous as well as homologous strains of pneumococci. (*c*) The components of the autolysates used as source of enzyme are themselves ineffective as capsular polysaccharide antigens.

It has been shown in a previous paper that by desiccation with cold acetone, it is possible to kill pneumococci under such conditions that their own autolytic enzymes remain present in an active form (2). When these desiccated cells are resuspended in a medium which destroys or inhibits the enzymes, the bacteria retain their characteristic morphology and staining reactions; in a physiological solution, on the contrary, the desiccated cells undergo autolysis immediately. Experiment 5 describes the comparative antigenicity in rabbits of pneumococci killed with acetone and resuspended in neutral buffer, or in iodine solution.

Experiment 5.—The cells collected from 300 cc. of broth culture of Pneumococcus Type I were resuspended in 10.0 cc. of cold distilled water, precipitated with 100 cc. of chilled acetone, washed again with acetone, and desiccated *in vacuo*. Half of the desiccated cells were resuspended in 10.0 cc. of M/20 phosphate buffer pH 6.9; this suspension kept for 5 minutes at room temperature, revealed only the presence of Gram-negative detritus. It was immediately heated at 70°C. for 20 minutes to prevent any further enzymatic action.

The other half of the desiccated material was resuspended in 10.0 cc. of M/100 iodine and maintained at 37°C. for 48 hours in this fluid. This suspension washed free of iodine was found to consist of Gram-positive cocci.

Two groups of three rabbits each were immunized with these two preparations; the animals were bled after the second course of injection. The presence of type specific precipitins was tested, using the sera in final dilutions of 1:2 and 1:5 and the Type I capsular polysaccharide in final dilution of 1:100,000.

The sera of the three rabbits immunized with the desiccated cells which remained intact and Gram-positive after iodination showed immediate precipitation in the presence of the type specific polysaccharide; on the other hand, no precipitin for the polysaccharide was demonstrable in the sera of the three animals which had received the desiccated cells allowed to autolyse in the neutral buffer solution.

It appears from the results of this experiment, that the acetone treatment does not *per se* inactivate the capsular polysaccharide anti-

gen, nor (as previously shown (2)) does it inactivate the autolytic enzymes; the antigenicity of the desiccated cells is lost only as a result of enzymatic action.

The activity of the enzymes present in the desiccated pneumococci was further demonstrated in the following manner. Type I pneumococci were killed with an amount of iodine sufficient to destroy irreversibly their autolytic enzymes (3), and then washed free of iodine. When acetone-desiccated cells were added to these iodinated cocci, the latter were reduced to Gram-negative detritus and, when injected into rabbits, failed to incite the production of precipitins for the type specific carbohydrates.

DISCUSSION

The capsular polysaccharides of pneumococci, long considered as true haptens, are now known to be antigenic in man, in mice, and in rats. Their antigenicity in mice, however, suffers some limitations hitherto unexplained. Casper and Schiemann (5), Avery and Goebel (6), have observed that when injected intraperitoneally into mice, these polysaccharides give rise to the development of active immunity only when used in very low concentration; when used in excess, the antigen is ineffective. At best, the protection induced in mice is somewhat erratic in contrast with the more uniform and greater immunity which follows immunization with the intact heat-killed cells.

Heat-killed pneumococci (Type I), which have been disintegrated with the pneumococcus bacteriolytic enzyme, still retain the property of inducing type specific active immunity in mice. The results presented in Experiment 1 indicate, however, that the enzyme-treated cells are not as efficient antigens as the intact cocci. In fact, the analogy between the antigenic activity in mice of the disintegrated cocci and of the free acetyl polysaccharide suggests that the residual antigenicity of the former material may be due solely to the presence of small amounts of free polysaccharide in the disintegrated cell suspension.

In the rabbit, the polysaccharides are not antigenic and fail to cause the production of precipitins, agglutinins, or protective antibodies, whatever the concentration or the route of injection used. It

is therefore apparent that in the intact bacterial cell, the capsular polysaccharide antigen is present in a form which differs in some way from the soluble products which have been isolated and purified.

The capsular polysaccharide antigen loses its efficacy in rabbits when the cells are disintegrated by autolysis, by treatment with bile, or by freezing and thawing. These three techniques involve the participation of some at least of the autolytic enzymes (2); in fact, it has been shown that heated or iodinated pneumococci that are capable of eliciting in rabbits the specific anticarbohydrate response lose this particular antigenic property after they have been exposed to the action of the active enzymes derived from homologous or heterologous strains of the organisms. This form of antigenic inactivation does not require that the cells have undergone lysis; it is sufficient that the enzymes render them Gram-negative, without destroying their characteristic morphology.

It is essential to point out once more that the present study is concerned only with the precipitins directed against the type specific capsular polysaccharides of encapsulated pneumococci. It is probable, however, that there exist in the pneumococcus cell other antigens which differ markedly from the capsular polysaccharide antigen in possessing greater resistance to cellular enzymes. But in any case, the paramount importance of the capsular polysaccharide antibodies in determining active and passive immunity to pneumococcus infections renders imperative the control of any autolytic process during the preparation of the cells to be used as immunizing agents.

SUMMARY

1. Mice immunized with heat-killed cells of virulent pneumococci (Type I) which have been treated with active preparations of the bacteriolytic enzyme, develop a certain degree of type specific resistance to subsequent infection. This active immunity, however, appears to be due to the small amount of free acetyl polysaccharide present in the suspension of digested bacteria, and is always of a less pronounced degree than that obtained with intact heat-killed cells.

2. Virulent pneumococci killed by heat or iodine when subjected to the action of active preparations of the bacteriolytic enzyme lose

the antigenic property of stimulating in rabbits the formation of precipitating antibodies for the type specific polysaccharide.

3. The enzyme prepared from S or R pneumococci, irrespective of type derivation, is equally effective against the capsular polysaccharide antigen of any specific type of this bacterial species.

4. The inactivation of the capsular polysaccharide antigen is observed when the cells are merely rendered Gram-negative, without being caused to undergo actual disintegration or lysis.

5. These observations emphasize the importance of minimizing the chances of alterations due to the action of cellular enzymes in the course of preparation of the cell suspension to be used as immunizing agents.

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PROPAGATION OF RABIES VIRUS IN TISSUE CULTURE

By LESLIE T. WEBSTER, M.D., AND ANNA D. CLOW

(From the Laboratories of The Rockefeller Institute for Medical Research)

It is generally agreed that rabies virus prior to 1936 had not been propagated *in vitro*. Levaditi's (1) and Imamura's (2) experiments indicated a survival but not an increase of virus; Noguchi's (3) findings have not been confirmed; Waldhecker (4) has reported only failures.

In October, 1936, however, Kanazawa (5) reported the cultivation of rabbit fixed virus in rabbit embryo brain plus Tyrode solution, and in March, 1937, published confirmatory data (6). His first communication was not convincing on the grounds that his media lacked serum, an omission which impairs cultivation of many viruses, including rabies (see below), and especially because he failed to show that his mice injected with later passage material were not succumbing to contaminants found in early passages but to rabies virus. The incubation period of his mice injected with culture material was less than that of mice given the original, supposedly virulent virus and less than that ordinarily encountered by others (Defries, 7; Webster, 8). Kanazawa's second report described a loss of virus after the tenth passage, its transfer and temporary survival in chick embryo brain, and a successful transfer back again to rabbit embryo brain and subsequent maintenance for a total of twenty-six passages. He reported cross serum protection tests demonstrating similar protective effects with culture and rabbit passage viruses, and noted similar encephalitic lesions in mice following injection.

In November, 1936, Webster and Clow (9) reported the propagation of rabies virus in mouse embryo brain plus serum-Tyrode media. They identified the flask culture with rabies virus by immunological tests and stated, furthermore, that the culture virus used as a vaccine protected mice against an intracerebral injection of street virus, and in dogs induced neutralizing antibodies promptly. The portion of this report dealing with cultivation of the virus will be described in this paper.

Technique

The method used to establish in tissue culture three strains of rabies virus was generally similar to that employed by Rivers and Ward (10) for the propagation of vaccine virus.

The origin of the three strains of virus was briefly as follows: Strain Sk_3P_6: Ammon's horn of a supposedly healthy skunk was injected into the brain of six mice (8). These animals developed typical rabies with Negri bodies on the 7th day. The brain of one prostrate mouse was removed, emulsified, and injected into two mice. In this manner the virus was passed serially through six mice and then inoculated into the culture flask. Strain R_1P_{88} was obtained from the brain of a rabid dog in New York and passed similarly through 88 mice. Strain $R_5_7P_8$ came from the brain of a rabid dog in Alabama and was passed by us through eight mice.

To Tyrode solution (NaCl, 8 gm.; KCl, 2 gm.; $CaCl_2$, 0.2 gm.; $MgCl_2$, 0.1 gm.; NaH_2PO_4, 0.05 gm.; $NaHCO_3$, 1 gm.; glucose, 1 gm.; distilled H_2O, 1,000 cc.) was added serum from normal *Macacus rhesus* monkeys in the proportion of 1 part to 9 parts of Tyrode. This mixture was filtered through a Seitz pad, the pH checked at approximately 8.2, and it was then placed in 4 cc. quantities in 50 cc. Erlenmeyer flasks.

Embryo tissue was prepared by decapitating a 14 to 20 day pregnant Swiss mouse, removing the uterus to a glass dish, washing it twice with saline, removing embryos with fresh instruments to a second glass dish, washing them twice with saline, and finally enucleating their brains with other instruments and transferring them to a watch glass in a Petri dish. Brains were washed once and emulsified as finely as possible with small, curved scissors. They were then weighed and diluted with 2 parts of serum-Tyrode to one part of tissue. The mixture was then added in 3 drop quantities (approximately 0.2 cc.) to the 4 cc. of serum-Tyrode in each culture flask.

Inoculation of rabies virus was made into the first flask by removing a virus-containing brain from a mouse prostrate following injection, emulsifying, and diluting it 1 to 1,000 with Tyrode. 1 cc. of this brain-virus suspension was then added to the flask of media. Flasks were then stoppered with tin-foil-covered corks and incubated 3 to 4 days at 37°C. Transfer to the second flask was made by removing the 4 day culture to a centrifuge tube, allowing it to settle or centrifuging it slowly, and then transferring 1 cc. of the resulting supernatant to the second flask of media. This method of serial inoculation of culture media was repeated regularly.

Cultures were run in triplicate. Sterile technique was used throughout and tests for sterility were made by inoculating 0.2 cc. of culture into hormone blood broth.

The quantity of virus present in a flask at a given period was determined by withdrawing 0.5 cc. of supernatant, diluting it with Tyrode, and injecting 0.03 cc.

of various tenfold dilutions intracerebrally into Swiss mice. The highest dilution giving a 50 per cent mortality was regarded as the minimum lethal dose.

Proof that the flask material was rabies virus was obtained by giving mice 0.25 cc. of the undiluted material intraperitoneally and testing them 3 weeks later for resistance to an intracerebral injection of known rabies virus. A second method was to test the ability of known rabies-neutralizing sera to protect mice against the culture virus.

RESULTS

A protocol showing the establishment of the SK_5P_6 strain in culture is shown in Table I. The greatest dilution of the original inoculated virus giving $50+$ per cent mortality was 10^{-5}; that of the first culture flask after 2 to 8 days' incubation, 10^{-2}. No change was observed for six serial transfers in flasks, after which the greatest effective dilution increased to 10^{-3} or 10^{-4} and has remained at that level for forty-two passages. Mice given the undiluted culture showed paralyses on the 7th or 8th days; those given the 1 to 1,000 dilution came down on the 10th to 12th days. This latter incubation period is twice that recorded by Kanazawa (5).

To identify the culture virus with rabies, a serum protection test was run with a known rabies-neutralizing serum plus control sera from normal persons and individuals immunized against St. Louis, louping ill, and Japanese encephalitis viruses. The rabies serum mixed with culture virus protected the injected mice against the usual 100 intracerebral lethal doses, while the other sera failed to protect against one lethal dose.

As an immunity test, nine mice were given an immunizing dose of thirteenth passage culture virus, 0.25 cc. intraperitoneally, and seven mice a similar dose of fifteenth passage virus. 3 weeks later these mice were tested for resistance to an intracerebral injection of known rabies mouse passage virus. Table II shows that normal mice succumbed to a 10^{-5} dilution of test virus, while the mice immunized with culture virus survived 1,000 times the fatal dose.

To check the amount of virus in a given flask at various intervals following inoculation, three flasks were each inoculated with 1 cc. of the eighteenth passage culture and titrated at various intervals. Later the test was repeated on three flasks inoculated with the twenty-fifth passage culture.

TABLE I

Propagation of Rabies Virus (Sk_3P_6) in Tissue Culture

Culture tested		Culture injected intracerebrally in 0.03 cc. amounts into two mice in dilutions						
Mouse brain virus		Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Passage flask	Days at 37°C.							
1st	2	—	—	6/9,* 6/9	7/9, 7/11	8/9, 8/9	†, 9/12	— —
"	4	8/9, 9/11	9/12, 12/14	8/11,				—
"	6	8/10, 8/10	8/11, 14/19	12/13, 12/14	10/12,			—
"	8	9/12, 10/12	8/12, 10/12	12/13, 12/13				—
4th	4	7/10, 8/10	9/11, 10/12	8/11				—
6th	4	7/11, 8/11	7/11, 8/11	11/13				—
8th	4	7/8, 8/10	7/10, 8/11	10/11, 8/11	13/15,	13/15,		—
9th	4	7/8, 7/8	7/8, 8/9	8/9, 9/9	11/12	—		—
10th	4	†, 7/8	7/8, 8/9	10/12, 10/13	11/12, 11/13			—
16th	4	—	8/12, 9/12	7/10, 10/11	10/11, 11/13	11/13		
25th	4	—	6/10, 6/10	8/11, 8/11	9/12, 11/12	12/13		
39th	4	6/9, 6/9	7/8, 7/9	7/11, 8/11	9/11, 10/12			—
42nd	4	6/7, 6/7	7/9, 7/10	6/7, 6/10	7/8, 8/11			—

* 6/9 = mouse paralyzed on 6th day and dead on 9th day.

— = dilution not tested.

Blank spaces indicate mice remained well.

† Mouse died of trauma within 12 hours of injection.

Text-fig. 1 shows that virus in the six flasks behaved relatively uniformly, being active in the 10^{-5} dilution on the 2nd day, 10^{-3} to

TABLE II

Antirabic Immunization of Mice with Culture Virus

Mice immunized with	Mice tested with standard virus 0.03 cc. intracerebrally in dilutions			
	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Nil-controls..............	—	6/7*, 6/7, 6/8	6/8, 6/8, 6/9	6/9, 8/9, 8/9
13th passage culture virus....	S, S, S	9/14, S, S	S, S, S	—
15th " " "	S, S, S	S, S	S, S	—

* 6/7 = mouse paralyzed on 6th day and dead on 7th day.

— = dilution not tested.

S = mouse remained well.
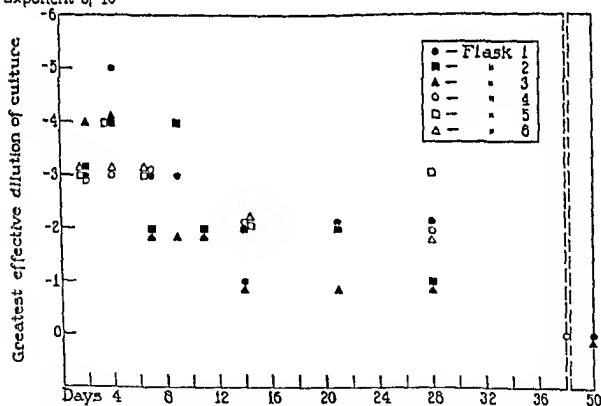
TEXT-FIG. 1. Titration of rabies virus in embryo mouse brain serum-Tyrode tissue culture.

10^{-5} on the 4th day, 10^{-2} to 10^{-3} on the 7th, 10^{-1} to 10^{-2} on the 14th day and remaining at this level for at least 4 weeks. At 50 days there was still active virus present in the two flasks tested.

The amount of inoculum per flask was reduced from 1 cc. to 0.5 cc. without altering the rate or amount of propagation of the established virus.

Monkey serum was replaced by horse serum without apparent effect on the culture virus. If serum was omitted from the medium, it was not adequate to propagate a newly introduced mouse brain virus, nor an already established culture virus.

Rabbit embryo brains were substituted for mouse embryo brains without alteration in titre of established culture virus.

Chick Embryo Brain Plus Monkey Serum-Tyrode.—Mouse embryo culture virus was readily established in chick embryo serum-Tyrode media (10).

The media and technique differed from that described above only in the substitution of chick embryo brains for embryo mouse brains. A 10 to 12 day chick embryo was removed aseptically from the egg, washed in sterile saline, and placed in a Petri dish. The brain was macerated in a mechanical grinder or with scissors and the resulting emulsion diluted 1 part to 2 parts of serum-Tyrode. 4 drops were added to each flask containing 4 cc. of serum-Tyrode.

After six and eight passages in the chick embryo culture media, the virus was active in the 10^{-3} dilution and readily identified as rabies.

Preservation.—The culture virus remained active in the ice box at $40°$ in fluid bulk or after freezing and drying. In both cases the virus was infective for 30 days when diluted 10^{-3}, and for at least 60 days when diluted 10^{-2}.

SUMMARY

Rabies virus has been propagated in serum-Tyrode solution containing either embryo mouse brain or embryo chick brain.

The culture virus reached a titre of 3×10^{-5} cc. after 4 days' incubation at $37°C.$, and survived at least 2 months at $5°C.$ in the liquid or dry state.

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THE MULTIPLICATION OF THE VIRUS OF YELLOW FEVER IN AËDES AEGYPTI*

By LORING WHITMAN, M.D.

(From the Laboratory of the Yellow Fever Service at Bahia, Brazil)

The question of whether or not yellow fever virus multiplies in *Aëdes aegypti* has long been a subject of debate. It is unfortunate that through lack of opportunity and material little work has been published on this subject.

Gay and Sellards (1) in 1927 stated their belief that such multiplication occurs, and they devised a hypothetical curve which proposed that there was first a drop in virus content followed by a rise. In 1932 Davis (2) showed that by increasing the temperature of the mosquitoes' environment, the period of incubation between the infection of *Aëdes aegypti* and their capacity to transmit by bite was shortened. Conversely, lowering the temperature lengthened this period. He believed that this was evidence of the multiplication of the virus in the mosquito. In 1933, however, Davis, Frobisher, and Lloyd (3) published experiments on the titration of virus in *Stegomyia* mosquitoes in which this opinion was reversed. By grinding up mosquitoes at intervals following their infection and injecting dilutions of them into monkeys, they showed that there was first a loss of virus followed by a rise. But as they were never able to recover subsequently as much virus as was present shortly after the mosquitoes were infected, they concluded that there had been no multiplication and that the incubation period could best be explained as the time required for the mechanical transportation of the virus to the salivary glands. Sellards (4) has reviewed Davis' papers, criticizing the conclusions of the latter studies and reaffirming his own conviction that multiplication in the insect host occurs. He offers no further evidence.

The possibility of virus multiplication in mosquitoes has received recent support in the experiments of Merrill and TenBroeck with the

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virus of equine encephalomyelitis (5). These authors have demonstrated that this virus can be passed from mosquito to mosquito without the intervention of an animal host. By feeding fresh batches of *Aëdes aegypti* on the ground bodies of mosquitoes previously infected, they have maintained the virus in undiminished quantity through more than 10 generations, thus proving that the virus has multiplied sufficiently in the bodies of the insects to compensate for the tremendous loss at each transfer.

Merrill and TenBroeck's method of demonstrating multiplication is conclusive. But it fails to show the fate of the virus from week to week in the bodies of each generation of mosquito and is applicable only to those species which feed readily on artificial mosquito-blood mixtures. It also requires a constant supply of insects over a long period of time. As our intention was to study eventually not only *Aëdes aegypti* but wild caught native mosquitoes as well, we decided upon the method of titration as being the most applicable to our needs.

All Davis' titrations were done in monkeys. This entailed considerable expense. Furthermore as the number of animals injected with each dilution of mosquito emulsion was small, the susceptibility or resistance of the individual test monkey influenced considerably the results of the experiments. Webster, Clow, and Bauer (6) have reported that mice can be used in titration studies on the fate of the virus of St. Louis encephalitis in *Anopheles quadrimaculatus*. This encouraged us to try these animals instead of monkeys, with the hope that by injecting 6 mice with each dilution of mosquito emulsion, a greater uniformity of results would follow. It would also permit us to carry out a larger number of observations.

In Davis' titration experiments the mosquitoes were always infected at a time when the monkeys on which they fed were known to have tremendous quantities of virus in circulation. The initial titre of the mosquitoes was therefore very high and the drop during the next few days marked. It seemed to us theoretically possible that in the biological relationship between virus and mosquito, the maximum titre obtainable following incubation might be lower than the artificially produced high titre demonstrated immediately after the ingestion of fully virulent monkey blood. It appeared logical to assume

that beyond a certain point, the growth requirements of the virus might surpass the capacity of the mosquito cells to supply them and that the maximum amount of virus present in the mosquito would be limited by this "supply and demand." Although Webster's results with *Anopheles* and the virus of St. Louis encephalitis have implied that there is a relationship between the initial and eventual titre of the mosquitoes, we hoped that by reducing the initial amount of virus in the mosquitoes, we could find a zone where sufficient virus was ingested by the mosquitoes to infect them all, but which would not yield titres too high to be surpassed should multiplication occur.

Methods and Materials

The *Aëdes aegypti* used in these experiments were raised in the laboratory. At the time of infection they were usually about 2 weeks old. They were kept in groups of about 200 insects each and were infected by introducing into the cage the source monkey on which they were to feed. The monkey was placed on a board and its abdomen shaved. ½ hour sufficed for nearly all the mosquitoes to engorge, at which time the monkey was withdrawn. When it was anticipated that many infected insects would be needed, the monkey was placed in a second cage, in which case the fed mosquitoes in the two cages were considered as one lot. After feeding, all non-engorged insects were removed and discarded. At subsequent intervals units of 30 mosquitoes were withdrawn for titration.

As mosquitoes vary in weight depending on the amount of water or honey they have recently taken, it was decided to adopt a standard weight of 2 mg. per mosquito rather than attempt to weigh them before each titration. Besides giving a constant basis for preparing diluents in advance, it saved the considerable time lost in weighing the insects after they had been anesthetized. The 30 mosquitoes to be titrated were chloroformed, and as soon as they ceased moving they were crushed in a mortar with 0.3 cc. of a human serum pool. This serum pool had been tested for the presence of substances injurious to yellow fever virus and was known to be free from them. To the crushed insects was added a small amount of sterile pyrex glass powder as an abrasive, and the whole was ground to a smooth paste. The glass powder had been prepared by cleaning in concentrated sulfuric acid and washing with distilled water until acid-free. It had then been dried and distributed in tubes for sterilization. After the mosquitoes had been ground to a paste, 2.64 cc. of a 10 per cent dilution of the above mentioned serum in normal salt solution was slowly stirred in. In this way 30 mosquitoes weighing 60 mg. were diluted with a total of 2.94 cc., making a 1:50 suspension by weight. This material was centrifuged for 5 minutes at low speed and the turbid supernatant fluid pipetted off for use, care being taken to avoid the film of fat which floats on the surface of the liquid. Serial tenfold dilutions of the 1:50 suspension were then made in the 10 per cent serum-saline diluent, a

fresh pipette being used for each transfer. 6 mice were injected intracerebrally with each of the dilutions, the volume of the inoculum being 0.03 cc.

To control the amount of virus originally taken up by the mosquitoes, the monkey was bled immediately after the insects had fed and the serum virus was titrated in mice. Serial tenfold dilutions were prepared, using the standard 10 per cent serum-saline diluent, and each of the dilutions was injected into 6 mice intracerebrally in 0.03 cc. amounts.

The Asibi strain of virus was used throughout these experiments.

In calculating the titres of both the monkey sera and the mosquitoes, a statistical method advocated by Muench was used (7). The end-point is considered as that dilution of virus which, when injected in 0.03 cc. amounts into

TABLE I

Titration of 30 Mosquitoes from Lot 5, 59 Days after Their Infection

Dilution of mosquitoes	Day of death of mice following inoculation	No. of mice		Cumulative No.		Dying
		Dying	Surviving	Dying	Surviving	
						per cent
1:50	8, 8, 8, 8, 9, 10	6	0	18	0	100
1:500	8, 8, 8, 9, 10, 11	6	0	12	0	100
1:5,000	10, 11, 11, 11, 13	5	1	6	1	85.7*
1:50,000	10	1	5	1	6	14.3*
1:500,000		0	6	0	12	0

* The difference between these two is 71.4. To reach a 50 per cent end-point, we need a dilution 35.7:71.4 of the distance between 1:5,000 and 1:50,000. This, calculated from a graph supplied us by Dr. Muench, is 1:16,000.

the brains of mice, will produce a 50 per cent mortality. In this paper we have used the reciprocal of the dilution instead of the dilution itself to signify the titre.

To clarify this method of determining the numerical end-point, Table I summarizes an actual titration.

Assuming that the mouse dying following the injection of the 1:50,000 dilution would have died if injected with the more concentrated suspensions and, conversely, that the mouse surviving the 1:5,000 dilution would have survived the more dilute suspensions, we can calculate the survivors and deaths as illustrated in Table I, thus smoothing out minor irregularities. From this we can calculate the exact end-point by means of Muench's graph.

EXPERIMENTAL

The first experiments were more or less exploratory and were not intended to be complete. It was our object to get a brief view of the

general response of the mosquitoes and to determine if possible the theoretical maximum concentration of virus in them.

Experiment 1.—Mosquitoes of lot 1 were allowed to feed on a *rhesus* monkey at the time when it first bad fever following the inoculation of yellow fever virus. After the mosquitoes had engorged, the monkey was bled and the serum titrated in mice. The titre was moderately high (495,000). Groups of 30 mosquitoes were withdrawn from lot 1 for titration 8, 16, 24, and 32 days after feeding on the monkey. The 8th day titre was 1,500, with a definite rise at each subsequent titration until at 32 days it had become 16,000. Table II gives the detailed results of this experiment.

TABLE II

Titres of Asibi Yellow Fever Virus in Aëdes aegypti at Intervals Following Their Infection

Time after feeding	Mosquito lots (Titres of monkey serum at time of feeding)		
	1 (495,000)	2 (1,800)	3 (1,000,000±)*
days			
3	—	0†	—
6	—	—	500
8	1,500	—	—
14	—	1,170	—
16	5,000	—	28,400
24	11,700	—	—
25	—	16,000	—
28	—	—	16,000
32	16,000	—	—

— = not tested.

* See text.

† No virus detectable in 1:50 dilution.

Experiment 2.—Mosquitoes of lot 2 were fed on a *rhesus* monkey on the 2nd day following its inoculation with source virus. At this time the monkey had no fever, and the titration of its serum showed that there was a very small amount of virus present in circulation, the titre being only 1,800. The mosquitoes were titrated 3, 14, and 25 days after their infectious meal. 3 days after the ingestion of this small amount of virus none could be demonstrated in the mosquitoes in the most concentrated suspensions tested (1:50). However, by 14 days virus was detectable with a titre of 1,170, and at 25 days it had increased to 16,000. Table II summarizes this experiment.

Experiment 3.—As in Experiment 2, mosquitoes of lot 3 were fed on a *rhesus* monkey on the 2nd day following its inoculation with source virus, and before the appearance of fever. In anticipation of a small quantity of virus in circulation, the series of dilutions tested was only carried to 1:10,000. This proved to be insufficient to reach the end-point of infectivity. Based on the average date of death of the mice following the injection of the several dilutions, it was estimated that the titre of the serum was approximately 1,000,000. The mosquitoes were titrated 6, 16, and 28 days after their infection. Starting with a titre of 500, the concentration of virus rose rapidly to 28,400 at 16 days, then fell to 16,000 at 28 days. Table II summarizes the first three experiments.

These first experiments demonstrated a relatively uniform titre of virus present in the mosquitoes following an incubation period of 3 to 4 weeks, regardless of the amount of virus taken up at the time of feeding. It indicated that if we could infect mosquitoes on amounts of virus so regulated that the initial titres were less than 16,000, yet which were sufficient to infect all the mosquitoes, tests for multiplication could be carried out. With this in mind the following experiments were completed.

Experiment 4.—Mosquitoes of lot 4 were fed on a *rhesus* monkey with a titre of circulating virus of 10,000. Within a few hours of feeding 30 of the mosquitoes were titrated as a basis for future comparison, and again at 4, 7, 14, 21, 28, 35 42, and 56 days. The titre a few hours after feeding was 2,150, but in 4 days it had fallen below detection in 1:50 dilution. At 7 days virus was again detectable and had regained its original titre at 2 weeks. Between 3 weeks and 8 weeks the titre fluctuated around 16,000, going as high as 21,500 and as low as 11,600. The average for this period was 17,230, a figure quite comparable to the results in the first three experiments. Table III gives the complete figures.

Experiment 5.—Mosquitoes of lot 5 were fed on a *rhesus* monkey with a titre of circulating virus of 4,000. They were titrated several hours after feeding, and at 5, 7, 14, 28, 59, and 70 days. The titre immediately after feeding was 1,600, and it fell by the 3rd day to undetectable amounts. On the 7th day enough virus was present in the 1:50 dilution to kill 1 of 6 mice injected. As this amount of virus is too small to determine mathematically we have estimated the titre as about 5. By 14 days the virus content had climbed to 3,500. In the 28 day titration the mice started to sicken more rapidly than in the preceding one, usually a sign that there is a greater quantity of virus in the inoculum, but as the final percentage of mice dying was only a little higher, the calculated titre was but 3,750. At 59 days, however, it had reached 16,000. About this time, owing both to the age of the mosquitoes and to the increasing heat and dryness of the environment, the remaining insects began to die. At 70 days barely enough mosquitoes were alive to complete a final titration, and the titre had fallen to 6,700. These results are summarized in Table III.

Experiment 6.—Mosquitoes of lot 6 were fed on a *rhesus* monkey with a titre of circulating virus of only 570. They were titrated several hours after feeding, and at 3, 7, 14, 42, and 56 days. The initial titre was 160. This dropped to less than a detectable amount and remained so for at least 2 weeks, and it was thought that the mosquitoes had not become infected. Unfortunately the author had to be away for several weeks following this and it was not possible to titrate the mosquitoes until the 42nd day. At that time virus had reappeared with a titre

TABLE III

Titres of Asibi Yellow Fever Virus in Aëdes aegypti at Intervals Following Their Infection

Time after feeding	Mosquito lots (Titres of monkey serum at time of feeding)		
	4 (10,000)	5 (4,000)	6 (570)
days			
0	2,150	1,600	160
3	—	0*	0*
4	0*	—	—
7	70	5±†	0*
14	2,150	3,500	0*
21	20,150	—	—
28	12,750	3,750	—
35	11,600	—	—
42	21,500	—	9,000
56	20,150	—	1,100‡
59	—	16,000	—
70	—	6,700§	—

— = not tested.

* No virus detectable in 1:50 dilution.

† Titre too low to be determined accurately. See text, Experiment 5.

‡ See text, Experiment 6.

§ See text, Experiment 5.

of 9,000. It would have been interesting to have determined when the virus first reached detectable quantities.

Because of the very low titre of the mosquitoes immediately after feeding, they were tested for their capacity to transmit by bite at the time the 14 day titration was made. A normal monkey was introduced into the cage and was fed upon by more than 200 insects. This monkey did not develop symptoms, nor was it immunized, showing that the mosquitoes had failed to transmit at that time. Immediately before the final titration at 56 days, another monkey was introduced into the cage. This monkey had been used in a previous experiment but was thought to be non-immune. Unfortunately it had become immunized, as was

proved by the demonstration of antibodies in the serum withdrawn just before the mosquitoes were fed on it. As a result, the titration of the engorged mosquitoes was complicated by the addition of the recently ingested immune serum. Since in the serial dilutions of the mosquito emulsions the immune serum was also being diluted, the effect was not marked in the more concentrated suspension. The 1:5,000 dilution was not particularly affected, as by this time the immune serum was too dilute to have any action. This dilution, as a matter of fact, had almost the identical effect on the mice as the same dilution in the 42 day titration. But the virus in both the 1:50 and 1:500 dilutions was partially neutralized, and there were delayed deaths and survivors in the mice injected with them. This reduced the number of mice dying in the titration as a whole and markedly lowered the titre to 1,100. Even so, this is more than 6 times higher than the initial reading. See Table III for the summary.

These experiments showed that, following an incubation period, titres of virus can be recovered from mosquitoes which are significantly greater than at any preceding time. This fulfills both Davis' and Webster's criterion of multiplication. However, in continuing this work we have found that the apparent uniformity in the response of the mosquitoes is subject to variation. In both previous series the final titre was constantly in the neighborhood of 16,000 to 20,000. Experiments conducted after an interval of approximately 6 months, in which *Aëdes aegypti* were used as controls for titrations on other native mosquitoes, showed that titres at least 10 times higher were to be observed. The following experiment may be taken as an example.

Experiment 7.—Two separate lots of *Aëdes aegypti* were fed at the same time on an Asibi-infected *rhesus* monkey at the height of infection. The serum of this monkey, titrated immediately after the mosquitoes had engorged, killed all of the mice injected with the 1:1,000,000 dilution. On the 3rd day 30 mosquitoes from each lot were titrated separately, yielding titres of 10,850 and 8,800. On the 14th day they were again separately titrated. At this time the quantity of virus in the mosquitoes was higher than was anticipated and a complete endpoint was not reached. In lot 7, however, 1 of 6 mice injected with the 1:50,000 dilution survived and 2 of 6 injected with 1:500,000, a total of 3 survivors in the titration. In lot 8, although no mice survived the 1:50,000 dilution, 3 of 6 mice survived the 1:500,000, again a total of 3 survivors in the titration. On the 16th day 3 groups of 30 mosquitoes each from lot 8 were titrated separately. The results of two titrations were 146,000 and 160,000. The third was identical with the 14th day titration from the same lot, namely, 3 of 6 mice survived in the 1:500,000 dilution. See Table IV.

This experiment shows a satisfactory correlation between the comparable titrations, but convincingly demonstrates a marked increase

in titre over the 16,000 level so constantly found previously. There are several ways to explain this phenomenon, but the one which appeared at the time to be the most likely was the effect of seasonal variations in temperature, rainfall, etc., on the capacity of the mosquitoes to multiply the virus. The earlier experiments had been done during the rainy season and following period of cool weather, while the more recent experiments were done during the hot dry season. It seemed possible that with higher prevailing temperatures greater quantities of virus might be expected to develop in the mosquito.

To test the effect of seasonal variations it was hoped that by waiting for weather conditions similar to those of the preceding year, we could

TABLE IV

Comparative Titres of Yellow Fever Virus in Two Lots of Aëdes aegypti Fed at the Same Time on the Same Infected Monkey

Time after feeding	Lot 7	Lot 8
days		
3	10,850	8,800
14	500,000±*	500,000±*
16		146,000
		160,000
		500,000±*

* See text, Experiment 7.

demonstrate that the titres would again be lower. Unfortunately the current year has been much dryer, and the rains, which have come late, have been less concentrated, so that we have not been able to duplicate the conditions of the earlier experiments. However, a final test was performed at about the same time of year as the second series of experiments.

Experiment 8.—400 mosquitoes of lot 9 were fed on an Asibi-infected *rhesus* monkey 65 hours after its inoculation and before the onset of fever. The titre of its serum at the time of the mosquito feeding was 57,000. 2 groups of 30 mosquitoes each were separately titrated on the day of feeding, the 3rd day, 24th day, and 38th day. The results of these titrations are summarized in Table V.

Although it is obvious that the comparable titrations do not check as well as those of Experiment 7, they nevertheless repeat the observa-

tions of the earlier experiments, namely that titres of virus well in excess of the initial level can be recovered from *Aëdes aegypti* infected with Asibi virus. This experiment fails to confirm the hypothesis of seasonal variation in the maximum virus contents of infected mosquitoes. However, in view of the fact that the seasons themselves have not been the same, this failure is not necessarily significant. It is quite possible that the low titres shown in the early experiments are the exceptions due to an unusually wet and cool season.

TABLE V

Titres of Yellow Fever Virus in Aëdes aegypti at Intervals Following Their Infection

Time after feeding	Lot 9	
	Titres obtained with 2 lots of 30 mosquitoes each	Average titre
days		
0	22,000 146,500	84,250
3	900 360	630
10	16,000 3,500	9,750
24	24,800 24,800	24,800
38	1,275,000 160,000	717,500

DISCUSSION

The experiments presented demonstrate that more virus can be recovered from *Aëdes aegypti* following an incubation period than immediately after feeding. These experiments also show that within limits this method of studying the fate of virus in mosquitoes is practical, but is subject to variations of unexplained origin. These variations may be the results of technical limitation, or may reflect factors of which we have little knowledge. It is believed that the change from the constant level of about 16,000 in the first two series

of experiments to the higher level in the last two is not due to chance or a change in technique but is an experimental fact. It is known by those who work with yellow fever virus that there are at times fluctuations in virulence of both the Asibi virus and other strains. Whether this is due to changes in the virus itself or merely reflects an altered physiological response on the part of the test animal is not known. It is possible that our higher titres in the last two experiments were due not to a change in environment as previously suggested but to just such a fluctuation in the virulence of the virus, or in the susceptibility of the mice used in the titrations. The fact that the last experiment shows discrepancies between the results of parallel titrations, particularly the first and last, probably reflects the errors of this method. Any single result is therefore subject to discount. When, however, as is shown here, series after series of titrations point in the same direction, we feel that we can ignore the error introduced by individual readings.

CONCLUSIONS

Aëdes aegypti have been shown to be capable of multiplying the Asibi strain of yellow fever virus in their bodies. Following the ingestion of infected blood, the content of virus falls for several days, reaching a minimum during the 1st week. It then increases rapidly until quantities of virus greater than those previously encountered can be demonstrated. The actual final amount of virus demonstrable, however, is subject to variations of which we know little.

We are grateful to Dr. P. C. A. Antunes for supplying us with the mosquitoes used in the studies here reported and for the maintenance of the infected insects.

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DOES LIVER SUPPLY FACTORS IN ADDITION TO IRON AND COPPER FOR HEMOGLOBIN REGENERATION IN NUTRITIONAL ANEMIA?*

By E. B. HART, C. A. ELVEHJEM, AND G. O. KOHLER

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison)

According to our present knowledge, the nutrients needed for maximum hemoglobin regeneration in uncomplicated secondary anemia are iron, copper, and amino acids for the construction of the protein part of the hemoglobin molecule. Since very rapid hemoglobin regeneration has been obtained in both rats and children on diets of whole milk plus iron and copper, we may conclude that milk was low only in these two elements as far as hemoglobin formation is concerned, and that the proteins in milk supply the amino acids essential for the production of the hemoglobin molecule. We should mention that milk may be low in manganese and an unknown organic factor, both of which are necessary for normal growth, but independent of hemoglobin formation.

In spite of these facts and probably because liver is such a valuable material for the treatment of pernicious anemia, great emphasis has been placed on the use of liver and liver products in secondary anemia. Minot and Castle (1) pointed out in 1931 that whole liver was not highly active in various types of human secondary anemia. Elvehjem (2), 1932, suggested that the limitations of liver in treatment of secondary anemia were probably due to the relatively low level of available iron supplied by most samples of liver.

However, Whipple, Robscheit-Robbins, and Walden (3) have described a secondary anemia fraction for liver which is active in hemoglobin regeneration in dogs suffering from hemorrhagic anemia. Sturgis and Farrar (4), using the technique described by Whipple and

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associates, found that liver promoted a definitely greater regeneration of hemoglobin than did the addition of an amount of inorganic iron equivalent to that contained in the added liver. They also showed that the response was no greater with iron plus casein than with iron alone which eliminates the possible protein effect of the liver. In spite of these facts high levels of inorganic iron have proved very effective in the treatment of a variety of hypochromic anemias. There is still some question about the reason for the need of such large amounts. Brock and Hunter (5) have shown that large doses are not needed for assimilation, and that when large amounts are fed the percentage utilization is very low. It is probable that the high intake of iron salts supplies added amounts of copper as a contamination, but this has not been demonstrated beyond question. Similarly in the experiments involving hemorrhagic anemia in dogs, no definite comparison has been made between the results obtained when whole liver is fed and both iron and copper are fed in amounts equivalent to that found in the liver. Whipple's diet contains some copper but according to the figures of Sturgis and Farrar the copper intake on the basal ration alone is quite low. The intake varies from 0.06 to 0.07 mg. per kilo of body weight per day. The requirement for a rat is at least 0.2 mg. per kilo. When liver was fed the intake of copper was raised to about 0.5 mg., which would be very close to the optimum intake. This may explain why Sturgis and Farrar obtained the increased hemoglobin regeneration with liver as contrasted with iron alone in hemorrhagic anemia.

We must also recognize that by bleeding many blood constituents are removed, the regeneration of which may require factors other than those needed for hemoglobin production.

In this paper we wish to emphasize that liver contains no factors in addition to iron and copper which can function as a supplement to milk for hemoglobin regeneration in rats.

EXPERIMENTAL

The usual technique was used for the production of the anemia, for feeding the animals, and for making the blood tests. The anemia was produced by restricting the young rats kept on galvanized wire screens to cow's milk. The records in most cases are the average of a number

of results obtained over a period of several years. Since records have been chosen from rats showing very similar growth responses, the growth curves have been eliminated and only the hemoglobin curves are presented. These curves are the composite record of four animals.

In Chart 1 results are given for rats receiving liver in addition to adequate amounts of iron and copper, for rats receiving liver as a
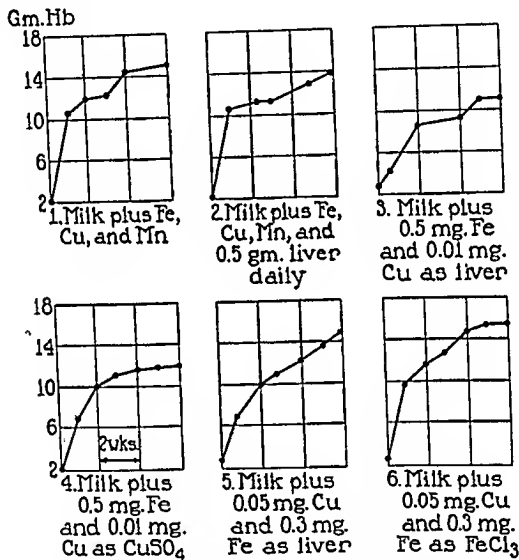
CHART 1. Curves showing the hemoglobin response in anemic rats on a milk diet when given liver in addition to iron, copper, and manganese, when given liver as a source of copper, and when given liver as a source of iron.

supply of copper, and for rats receiving liver as a supply of iron. In all cases the liver was obtained directly from the packing plant and dried at 65°C. Both beef and hog liver have been used. Curve 1 shows the typical response of rats receiving 0.5 mg. Fe, 0.05 mg. Cu, and 0.05 mg. Mn daily in addition to whole milk. Hundreds of

curves of this type have been obtained during the past 7 years. Curve 2 illustrates the average response of ten animals which received the same mineral supplement plus 0.5 gm. dry beef liver per day. The results are identical. Thus the liver produced no change in the course of regeneration. The remaining curves show that liver can serve as a source of both iron and copper when the intake of these elements from the basal diet is limited. Curves 3 and 4 are taken from a recent paper by Schultze, Elvehjem, and Hart (6). The rats in both groups received 0.5 mg. Fe daily. Curve 3 shows the response in the rats receiving 0.01 mg. Cu in the form of hog liver and Curve 4 shows the response in those receiving 0.01 mg. as $CuSO_4$. The results are very similar and show that when copper is the limiting factor the effectiveness of liver is directly proportional to its copper content. The response in either case is not optimum because of the low level of copper used. Curves 5 and 6 show similar results in the case of iron. These rats received 0.05 mg. Cu per day. Curve 5 illustrates the response obtained in rats receiving 0.3 mg. total iron from beef liver (1.15 gm. dry basis) and curve 6 the response when 0.3 mg. of $FeCl_3$ was fed daily. Again the response is very similar although the regeneration in the rats receiving the liver was somewhat retarded the first 2 weeks. This can be accounted for by the fact that not all the iron in liver is available to the rat. This sample of liver contained 70 per cent of the total iron in available form. When iron is the limiting factor in the basal ration, the value of liver depends upon its available iron content.

A very large number of results of this type could be presented. They all show that the value of liver in the treatment of nutritional anemia depends directly upon the amount of available iron and copper which the liver can supply. Very recently several products have been placed on the market in which iron or iron and copper preparations have been combined with whole liver, liver extract, or other liver preparations. Liver extract is, of course, very low in iron but does contain some copper. In fact liver extract was one of the products used in the early experiments which demonstrated the essential nature of copper. In order to determine if these combinations have any virtue in simple hemoglobin regeneration beyond their copper and iron content, several preparations were purchased on the market, analyzed for iron and copper, and fed to rats at levels sufficient to

supply 0.5 mg. Fe daily. Typical results for four of these prepara-
tions are given in Chart 2. Results for control rats receiving 0.5 mg.
Fe and 0.05 mg. Cu daily are included for comparison. Preparations
A and C gave responses very similar to the controls. In both cases
the daily dose necessary to supply 0.5 mg. Fe contained about 0.02
mg. Cu, a level which is sufficient to give good regeneration. Sample
B gave a fairly good response and supplied 0.008 mg. copper daily,
which is below the needed amount. The response with sample D was
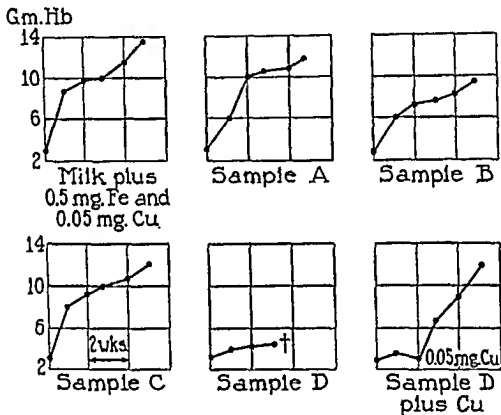
CHART 2. Hemoglobin response in anemic rats on milk diet supplemented with
various commercial liver preparations when fed at levels which supplied 0.5 mg.
Fe daily.

practically negative due to the low copper intake of 0.0005 mg. daily.
Further evidence that this is a true copper deficiency is shown in the
next curve, where 0.05 mg. copper is added after 2 weeks on the
preparation alone. An immediate response resulted when the copper
was added. Here again the potency of these products for the cure
of nutritional anemia in rats is directly related to the iron and copper
content and the ratio of one element to the other. The presence of
the added liver products adds nothing to their value for this particular
purpose.

These facts do not detract from the nutritional value of liver extract, for there is ample evidence in the literature that this product is a most excellent source of most of the factors in the B complex, but they do emphasize the importance of recognizing the value and limitations of each ingredient in such mixtures. In the case of anemia due to a simple deficiency of iron and copper, which is the case in many children, small doses of iron and copper work very efficiently with no other additions. When milk constitutes a large part of the diet it supplies ample amounts of the other nutrients needed for hemoglobin formation. In more complicated deficiencies the anemia should be separated from the other disturbances. If the additional disturbances are due to a lack of certain of the B vitamins, the pernicious anemia factor, or protein, liver extract may be used very efficiently. Each material should stand on its own merits and be used only when there is a need of the nutrients which it supplies. False association or the so called shotgun therapy should be discouraged.

SUMMARY

1. Our data indicate that the effectiveness of whole liver in the treatment of nutritional anemia in rats induced by a milk diet is directly proportional to its available iron and copper content. The other constituents in liver are not needed for maximum hemoglobin regeneration on a diet of milk, iron, copper, and manganese.

2. Commercial preparations of liver products with iron or iron and copper vary greatly in their hemoglobin-regenerating efficiency in rats with nutritional anemia. The variation is correlated directly with the iron and copper content of the preparation. When the copper-iron ratio was too wide hemoglobin regeneration was checked, although the iron supply was sufficient for optimum regeneration.

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IMMUNOLOGICAL RELATIONSHIP BETWEEN THE SWINE AND HUMAN INFLUENZA VIRUSES IN SWINE

By RICHARD E. SHOPE, M.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

Ferrets or mice recovered from infection with the virus of human or swine influenza are usually immune to infection with the other virus (1–3). In these two animals a complete and frequently fatal disease is produced by either type of influenza virus alone, and there is no evidence that concomitant infection with *Hemophilus influenzae suis* or any other bacterium modifies its course in any constant manner (1, 3–6). Swine, on the other hand, infected with either swine or human influenza virus alone develop but a mild, transient, indefinite illness (filtrate disease) and come down with influenza only when the bacterium, *H. influenzae suis* (7), has accompanied the virus (8–10). It seemed possible that the cross-immunological relationship between swine and human influenza virus found in the simple virus infections of ferrets and mice might not follow in the complex virus-bacterium infections necessary to induce influenza in swine. The present paper reports experiments dealing with the cross-immunization of swine by means of initial infections with either swine or human influenza virus alone or in mixture with the bacterium, *H. influenzae suis*.

EXPERIMENTAL

Infectious Materials Used

Francis' P.R. 8 strain (5) human influenza virus and strain 15 (Iowa, 1930) swine influenza virus were employed in all experiments. Culture 18 (11) *H. influenzae suis* was used to complete the etiological complex with either strain of virus in most cases, although in a few instances this was pooled with cultures 23 and 24, more recently isolated from field cases of swine influenza.

Virus, either the human or the porcine type, was in all experiments prepared in physiological saline as a 10 per cent suspension of lung from swine infected with virus alone. The swine strain had originally been freed of *H. influenzae suis* by

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Berkefeld filtration or by serial passage through ferrets or mice. Swine whose infections were to be with virus alone were given from 6 to 10 cc. of the supernatant fluid from sedimented but uncentrifuged suspensions intranasally. Swine whose infections were to be with a mixture of virus and bacterium received, in addition to virus, 0.5 to 1 cc. of a 24 hour horse blood culture[1] of *H. influenzae suis*. The culture was mixed with the virus suspension just prior to its administration intranasally. Variations in the dosage of either virus or bacterium, within the limits used in the present experiments, had no influence on the results obtained.

Immunity to Swine Influenza Induced by Infection with Human Influenza Virus Alone or in Mixture with Hemophilus influenzae suis

Eight swine were inoculated intranasally with a mixture of human influenza virus and *H. influenzae suis*. As noted in Table I, 6 of these animals developed an illness that was clinically characteristic of a mild swine influenza. The remaining 2 came down with an illness which clinically resembled that produced in swine by infection with virus alone, and it is believed that in these *H. influenzae suis* failed to become established with the virus in the respiratory tract. The occasional failure of this bacterium to establish itself with human influenza virus in the swine respiratory tract is well known from earlier work (10).

Nine swine inoculated intranasally with human influenza virus alone developed the mild, indefinite, filtrate disease. 2 other swine receiving human influenza virus alone intranasally twice at 20 day intervals exhibited symptoms of filtrate disease following the first inoculation only.

When the swine had completely recovered from their human influenza infections they were tested for immunity to swine influenza by inoculating them intranasally, together with control swine, with a mixture of swine influenza virus and *H. influenzae suis*. The results of these tests for immunity are outlined in Table I.

As shown in the table, 6 of the 8 swine whose initial infection had been with a mixture of human influenza virus and *H. influenzae suis* proved immune to swine influenza. Of the remaining animals, swine 1820 developed a transient fever but did not appear ill, while the other one, swine 1823, whose initial infection had clinically resembled filtrate disease, was febrile and depressed and exhibited a scattered lobular pneumonia when autopsied on the 3rd day. Swine influenza virus was demonstrated, by mouse inoculation, in the lung of this animal although its presence could not be demonstrated in the turbi-

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[1] 0.5 to 1 cc. of sterile defibrinated horse blood added to a plain agar slant. In this medium *H. influenzae suis* grows largely in the blood at the base of the slant with only scant colony formation on the agar surface.

nates. *H. influenzae suis* could not be cultivated from either the lung or terminal bronchi.

Four of the 6 swine that had appeared clinically immune to swine influenza were killed and autopsied on the 3rd or 4th day after inoculation. No lesions of swine influenza were seen in their respiratory tracts. Their lungs appeared normal aside from scant, old, puckering scars in the anterior lobes, evidently residual for their initial human influenza infection. Virus could not be demonstrated by mouse inoculation in the lungs of any of the animals nor in the turbinates of 2 tested. Neither could *H. influenzae suis* be cultivated from their lungs or terminal bronchi. Autopsy thus confirmed the clinical evidence that these 4 swine had been immune to swine influenza. The remaining 3 of the 8 swine initially infected with human influenza virus and *H. influenzae suis* were kept under observation in order later to obtain serum for neutralizing antibody studies.

The results obtained in the swine whose initial infections had been with human influenza virus alone differed from those just described. Only 1 animal, swine 1780, proved completely immune to swine influenza. The remaining 8 developed disease varying clinically from that seen in normal swine infected with swine influenza to that in which the salient features were merely a transient depression with or without fever. 6 of these animals were killed and autopsied on the 3rd or 4th day. One, swine 1729, showed no influenzal pneumonia; 1, swine 1747, showed only a pleuritis; while, in the remaining 4, pneumonias of from 1 to 3 lobes were encountered. These pneumonias were qualitatively like those seen in the control animals but were in most cases less extensive. However, although swine influenza virus was regularly detectable by mouse inoculation in the turbinates and lungs of the control swine, it was either not demonstrated or present only in low concentrations in the turbinates and lungs of the human virus-immune animals. *H. influenzae suis* could be cultivated from the lungs of 4 of the 6 swine autopsied and from the terminal bronchi of all. Its presence in this group of animals was in striking contrast to its uniform absence in the lungs and terminal bronchi of the swine whose initial infection had been with a mixture of human virus and *H. influenzae suis.*

The 2 swine that had been inoculated intranasally twice at 20 day intervals with human influenza virus alone were found clinically

TABLE I

Immunity to Swine Influenza Induced by Infection with Human Influenza Virus Alone or in Mixture with Hemophilus influenzae suis

Swine No.	Human influenza infection		Time between infections	Test for immunity to swine influenza					
	Inoculated intranasally with	Result		Clinical illness	Findings at autopsy				
					Lung lesions	Virus in		H. influenzae suis in	
						Turbinates	Lung	Terminal bronchi	Lung
			days						
1599	Human influenza virus + H. influenzae suis	Mild influenza	10	None	Scant and old	Not tested	Absent	Absent	Absent
1605	"	"	10	"	" "	" "	"	"	"
1714	"	"	13	"	" "	Absent	"	"	"
1739	"	"	13	"	" "	"	"	"	"
1823	"	"Filtrate disease"	13	Fever and depression	Scattered lobular pneumonia	"	Present	"	"
1720	Human influenza virus alone	"	13	Mild influenza	1 lobe pneumonia	"	Absent	Present	Present
1729	"	"	13	Depression, no fever	Scant and old	Present	"	"	Absent
1742	"	"	12	Typical influenza	2 lobe pneumonia	"	Present	"	Present
1746	"	"	12	Mild influenza	3 lobe pneumonia	Absent	Absent	"	"
1747	"	"	12	Fever and depression	Bilateral pleuritis	Present	"	"	"
1779	"	"	12	Depression, no fever	Scant pneumonia	Absent	Present	"	Absent
1662	Nil, control			Typical influenza	5 lobe pneumonia	Not tested	Not tested	"	Present

No.	Treatment	Interval (days)							
1664	"		"	"	3 lobe pneumonia	Present	"	"	"
1693	"		"	"	5 lobe pneumonia	"	Present	"	"
1698	"		"	"	4 lobe pneumonia	Not tested	Not tested	"	"
1784	"		"	Mild influenza	3 lobe pneumonia	Present	Present	"	"
1717	"		"	Typical influenza	4 lobe pneumonia	"	"	"	"
1763	"		"	"	3 lobe pneumonia	"	"	"	"
1830	"		"	"	5 lobe pneumonia	"	"	"	"
1819	Human influenza virus + H. influenzae suis	13	Filtrate disease	None	Not autopsied, see Table III				
1820	"	13	Mild influenza	Transient fever, not ill					
1821	Human influenza virus alone	13	Filtrate disease	None					
1645	"	20	"	Fever and depression					
1657	Human influenza virus alone twice at 20 day interval	11	Filtrate disease after 1st inoculation	None					
1659	"	11	"	"					
1750	Human influenza virus alone	12	Filtrate disease	Mild influenza					
1780	"	12	"	None					

* Sera obtained at end of this interval neutralized the human but not the swine influenza virus in all cases.

† Intranasal inoculation with mixture of swine influenza virus and *H. influenzae suis*.

immune to swine influenza when later tested. They, together with 3 swine receiving a single injection of human influenza virus prior to testing for immunity to swine influenza, were kept under observation in order subsequently to obtain serum for neutralizing antibody studies.

It would appear from these experiments that, while initial infection with a mixture of human influenza virus and *H. influenzae suis* usually immunizes swine to swine influenza, initial infection with the human virus alone usually fails to do so, although it does appreciably alter their susceptibility. That the cross-immunity to swine influenza conferred by a primary infection with the human agent is not associated with demonstrable virus-neutralizing antibodies for the swine virus is indicated by the fact that the sera of all 19 swine studied, obtained just prior to the inoculation test for immunity to swine influenza, failed to neutralize the swine agent. All, however, neutralized the human virus completely.

Technique of the Neutralization Tests.—The neutralization tests recorded throughout this paper were conducted in the usual way in mice (12), employing the supernatant of a 2 per cent suspension of infected mouse lung as virus and mixing this in equal parts with the undiluted sera to be tested. Either 3 or 4 mice, while under ether narcosis, were inoculated in each test by dipping their noses in the virus-serum mixture contained in a slightly tilted Petri dish. Surviving mice were killed on the 7th day and their lungs, together with those of mice dying earlier, were examined for the presence of influenza lesions. Mice which survived 7 days and whose lungs showed no influenzal pneumonia at autopsy were considered to have received a completely neutralizing serum, mice which survived 7 days but whose lungs showed influenzal lesions at autopsy were considered to have received a partially neutralizing serum, while mice which died of an influenzal pneumonia during the period of observation were considered to have received a non-neutralizing serum. The swine and human viruses employed in the neutralization tests were of such virulence as to kill all control mice within 7 days.

Immunity to Human Influenza Infection[2] Induced by Infection with Swine Influenza or Swine Influenza Virus Alone

Six swine inoculated intranasally with a mixture of swine influenza virus and *H. influenzae suis* developed swine influenza. 8 swine inoculated intranasally

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[2] In order to simplify terminology, "human influenza infection" is used to indicate an infection with a mixture of human influenza virus and *H. influenzae suis*.

with swine influenza virus alone came down with filtrate disease. Following complete recovery all 14 animals were tested for immunity to human influenza infection by inoculating them intranasally, together with control swine, with a mixture of human influenza virus and *H. influenzae suis*. The results of these tests for immunity are given in Table II.

As shown in the table, all 6 of the swine initially infected with swine influenza proved clinically immune to human influenza infection. 2 of these animals were killed and autopsied on the 4th day after inoculation. No lesions of human influenza infection were seen in their respiratory tracts and their lungs appeared normal aside from old healing lesions in the anterior lobes, residual from the initial swine influenza infections. Virus could not be demonstrated by mouse inoculation in the lungs or turbinates and *H. influenzae suis* could not be cultivated from either the lungs or terminal bronchi. Clinical evidence of immunity was thus confirmed by postmortem findings. The remaining 4 swine in the group were saved for later neutralizing antibody studies.

Of the 8 swine initially infected with swine influenza virus alone, 6 proved clinically immune to later human influenza infection. The remaining 2 became ill, but in neither of these were the postmortem findings characteristic of a human influenza infection. One animal (swine 1778) showed no recent respiratory tract lesions at all, merely an old, unresolved, scattered, lobular pneumonia probably persisting since the initial swine virus infection. The other animal (swine 1673) had a bilateral fibrinous pleuritis and pericarditis and from the exudate *H. influenzae suis* and a streptococcus were cultivated. 2 of the clinically immune animals killed and autopsied 4 days after inoculation showed no lesions of human influenza infection. In the anterior lobes of the lungs of both animals were scant contracted old scars evidently the result of healing swine influenza virus lesions. Virus could not be demonstrated by mouse inoculation in the turbinates or lungs of any of the 4 swine autopsied. The remaining 4 swine in the group, all clinically immune to human influenza infection, were kept under observation for later neutralizing antibody studies.

It is apparent from these experiments that initial infection with both the agents responsible for swine influenza or the swine influenza virus alone usually immunizes swine to human influenza infection, and

TABLE II

Immunity to Infection with Mixture of Human Influenza Virus and Hemophilus influenzae suis Induced by Infection with Swine Influenza or Swine Influenza Virus Alone

Swine No.	Swine influenza infection		Test for immunity to human influenza infection†					
	Inoculated intranasally with	Result	Time between infections	Clinical illness	Findings at autopsy			
						Virus in		H. influenzae suis in
			days		Lung lesions	Turbinates	Lung	Terminal bronchi	Lung
1723	Swine influenza virus + H. influenzae suis	Typical influenza	13	None	Old and healing	Absent	Absent	Absent	Absent
1815	"	Mild influenza	19	"	" "	"	"	"	"
1673	Swine influenza virus alone	Filtrate disease	15	Fever and prostration	Bilateral pleuritis	"	"	"	Present
1727	" " "	" "	16	None	Scant old scars	"	"	"	Absent
1776	" " "	" "	12	"	" "	"	"	Present	"
1778	" " "	" "	12	Transient fever	Scattered old lobular pneumonia	"	"	Present	Present
1691	Nil, control			Mild influenza	1 lobe pneumonia	Present	Present	"	"
1672	" "			Filtrate disease	Scant, scattered and lobular	"	"	Absent	Absent
1741	" "			Mild influenza	1.5 lobe pneumonia	"	"	Present	Present
1793	" "			" "	" "	"	"	"	"

				Filtrate disease	Not autopsied
1819	"			Mild influenza	Not autopsied
1820	"			"	"
1821	"			"	"
1678	Swine influenza virus + H. influenzae suis	Mild influenza	18	None	
1683	"	Typical influenza	15	"	
		" "	15	"	
1787	"	Mild influenza	19	"	
1801	"	"	15	"	Not autopsied, see Table III
1665	Swine influenza virus alone	Filtrate disease	15	"	
1668	"	" "	15	"	
1744	"	" "	12	"	
1775	"	" "	12	"	

* Sera obtained at end of this interval neutralized the swine but not the human influenza virus in all cases except that of swine 1744 (see Table III).

† Intranasal inoculation with mixture of human influenza virus and H. influenzae suis.

TABLE III

Influence of Initial Virus Infection upon Subsequent Antibody Response to the Viruses of Human and Swine Influenza

Swine No.	Serum drawn	Serum tested for capacity to neutralize							
		Swine influenza virus				Human influenza virus			
		Extent of pulmonary lesions in mouse No.				Extent of pulmonary lesions in mouse No.			
		1	2	3	4	1	2	3	4
	(a) Initial infection. Human influenza virus: Reinoculated with swine influenza virus intranasally								
1819	Normal	4+*	4+	4+	4+	4+	4+	4+	4+
	12 days after initial infection	4+	4+	4+	3+	0	0	0	0
	12 days after reinoculation	0	0	0	0	0	0	0	0
1820	Normal	4+	4+	4+	4+	4+	4+	4+	4+
	12 days after initial infection	4+	4+	4+	3+	0	0	0	0
	12 days after reinoculation	2+	1+	1+	0	0	0	0.	0
1821	Normal	4+	4+	4+	4+	4+	4+	4+	
	12 days after initial infection	4+	4+	4+	4+	0	1+	0	0
	12 days after reinoculation	0	0	0	0	0	0	0	0
1645	Normal	4+	4+	4+	4+	4+	4+	4+	4+
	19 days after initial infection	4+	4+	4+		0	0	0	0
	11 days after reinoculation	0	0	0	0	0	0	0	0
1657	Normal	4+	4+	4+	4+	4+	4+	4+	4+
	31 days after initial infection*	4+	4+	4+	4+	0	0	0	0
	11 days after reinoculation	0	0	0	0	0	0	0	0
1659	Normal	4+	4+	4+	4+	4+	4+	4+	4+
	31 days after initial infection*	4+	4+	4+	4+	0	0	0	0
	11 days after reinoculation	0	0	0	0	0	0	0	0
1750	Normal	4+	4+	4+	4+	4+	4+	4+	4+
	12 days after initial infection	4+	4+	4+	4+	0	0	0	0
	11 days after reinoculation	0	0	0	0	0	0	0	0
1780	Normal	4+	4+	4+	4+	4+	4+	4+	4+
	12 days after initial infection	4+	4+	4+	4+	0	1+	0	0
	11 days after reinoculation	4+	4+	4+	4+	0	0	0	0

* 0 = mouse with no pulmonary lesions at autopsy. 1+ to 4+ = mice with progressive degrees of influenzal pneumonia; 4+ indicates a complete and fatal pneumonia.

TABLE III—*Concluded*

Swine No.	Serum drawn	Serum tested for capacity to neutralize							
		Swine influenza virus				Human influenza virus			
		Extent of pulmonary lesions in mouse No.				Extent of pulmonary lesions in mouse No.			
		1	2	3	4	1	2	3	4
(b) Initial infection. Swine influenza virus: Reinoculated with human influenza virus intranasally									
1678	Normal (not obtained)								
	22 days after initial infection	0	0	0	0	4+	4+	4+	4+
	12 days after reinoculation	0	0	0	0	0	0	0	0
1683	Normal	4+	4+	4+		4+	4+	4+	4+
	13 days after initial infection	0	0	0	0	4+	4+	3+	3+
	12 days after reinoculation	0	0	0	0	4+	3+	3+	2+
1787	Normal	4+	4+	4+	4+	4+	4+	4+	4+
	13 days after initial infection	0	0	0	0	4+	4+	4+	2+
	12 days after reinoculation	0	0	0	0	2+	2+	2+	2+
1801	Normal (not obtained)								
	16 days after initial infection	0	0	0	0	4+	4+	4+	4+
	12 days after reinoculation	0	0	0	0	4+	4+	4+	4+
1665	Normal	4+	4+	4+	4+	4+	4+	4+	4+
	14 days after initial infection	0	0	0		4+	4+	4+	4+
	12 days after reinoculation	0	0	0	0	1+	1+	1+	1+
1668	Normal	4+	4+	4+	4+	4+	4+	4+	4+
	14 days after initial infection	0	0	0		4+	4+	4+	3+
	12 days after reinoculation	0	0	0	0	4+	3+	3+	2+
1744	Normal	4+	4+	4+	4+	4+	4+	4+	4+
	12 days after initial infection	0	0	0	0	3+	2+	2+	1+
	11 days after reinoculation	0	0	0	0	3+	2+	2+	2+
1775	Normal	4+	4+	4+	4+	4+	4+	4+	4+
	12 days after initial infection	0	0	0	0	4+	4+	4+	3+
	11 days after reinoculation	0	0	0		4+	4+	4+	3+

that the virus alone is little if any less effective in achieving immunity than is a mixture of virus and *H. influenzae suis*. The cross-immunity to human influenza infection conferred by the porcine agent is not

usually associated with demonstrable virus-neutralizing antibodies for the human virus. Of the 14 swine studied, the serum of only 1 (swine 1744), obtained just prior to the test for immunity to human influenza infection, exerted any neutralizing effect on the human virus. The remaining 13 sera, although neutralizing swine virus completely, were devoid of neutralizing activity for the human agent.

Influence of Initial Virus Infection upon Subsequent Antibody Response to the Viruses of Human and Swine Influenza

In order to determine whether swine would develop neutralizing antibodies for either swine or human influenza virus, when inoculated intranasally with these agents following recovery from an initial infection in which the heterologous virus had been employed, the following experiments were carried out.

Eight swine recovered from infection with either human influenza virus alone or a mixture of human influenza virus and *H. influenzae suis* were reinoculated intranasally with a mixture of swine influenza virus and *H. influenzae suis*. Some proved clinically immune and others not, as recorded in Table I. 11 or 12 days after reinoculation they were bled and the serum then obtained, together with that secured before and following recovery from their initial infection, was tested for the presence of neutralizing antibodies for the swine and human viruses by the usual technique (12).

Eight further swine recovered from infection with swine influenza or swine influenza virus alone were reinoculated intranasally with a mixture of human influenza virus and *H. influenzae suis*. All proved clinically immune, as recorded in Table II. Like those in the preceding group, they were bled 11 or 12 days after reinoculation and the serum obtained, together with that drawn before and following recovery from their initial infection, was tested for neutralizing antibodies against both viruses. The results of the tests of these 2 groups of swine sera are outlined in Table III. Since the development of neutralizing antibodies for either swine or human influenza virus was independent of whether or not *H. influenzae suis* had accompanied the virus in the infection, no distinction is made in the table between the animals initially infected with virus alone and those infected with a mixture of virus and bacterium.

As shown in the table, it was found that the sera of all 8 swine, obtained following recovery from an initial infection with human influenza virus, neutralized the human but not the swine agent. Reinoculation of these animals intranasally with swine influenza virus resulted in the appearance, in sera obtained 11 or 12 days later, of

antibodies neutralizing the swine virus completely in 6 of the 8 cases. In the serum of 1 animal (swine 1820) a weaker titer of swine virus antibody appeared, while in the serum of the remaining animal (swine 1780) no swine virus-neutralizing antibodies were demonstrated. Antibodies developed independently of whether or not the animals exhibited recognizable clinical manifestations of infection following reinoculation with swine influenza.

The results obtained in studies with sera of swine initially infected with swine influenza virus and reinoculated intranasally with the human agent were quite different from those just described. Only 1 (swine 1678) of the 8 swine developed antibodies which completely neutralized the human virus. 2 others (swine 1667 and 1787) developed antibodies which neutralized partially under the conditions of the test. The sera of the remaining 5 swine failed to show a significant increase in neutralizing antibodies for the human virus. Swine 1744, whose serum drawn before reinoculation with the human virus partially neutralized, still only partially neutralized afterwards.

It seems clear from the experiments just described that the swine and human influenza viruses influence the subsequent immunological reactivity of swine in differing fashions. To summarize, swine recovered from infection with swine influenza virus are not only immune to the human influenza virus but usually fail to develop specific virus-neutralizing antibodies for it following intranasal inoculation. Swine recovered from initial infection with human influenza virus, on the other hand, may or may not prove immune to swine influenza, but whether or not immune, usually elaborate swine influenza virus-neutralizing antibodies.

The Antibody Response of Swine Influenza-Convalescent Swine to Human Influenza Virus Administered Intramuscularly

There were two obvious possible explanations for the general failure of swine influenza-recovered swine to develop neutralizing antibodies for the human influenza virus following intranasal inoculation. First, the immunity conferred by a previous infection with the swine virus might be of such a nature as to render the respiratory tract mucosa actually impermeable to the human virus. If this were the case and human virus were completely prevented from invading susceptible

cells, one should not expect an antibody response.   Second, previous infection with swine virus might, in some manner, have interfered with or exhausted the mechanism responsible for the elaboration of neutralizing antibodies for the closely related human virus.   In this

TABLE IV

Antibody Response of Swine Influenza-Convalescent Swine to Human Influenza Virus Administered Intramuscularly

Swine No.	Serum drawn	Serum tested for capacity to neutralize							
		Swine influenza virus				Human influenza virus			
		Extent of pulmonary lesions in mouse No.				Extent of pulmonary lesions in mouse No.			
		1	2	3	4	1	2	3	4
Initial infection. Swine influenza virus: Reinoculated with human influenza virus intramuscularly									
1893	Normal	4+*	4+	4+		4+	4+	3+	
	13 days after initial infection	0	0	0		1+	2+	1+	
	11 days after reinoculation	0	0	0		1+	0	1+	
1894	Normal	4+	4+	4+		4+	4+	4+	
	13 days after initial infection	0	0	0		4+	4+	4+	
	11 days after reinoculation	0	0	0		0	0	0	
1895	Normal	4+	4+	4+		4+	4+	4+	
	13 days after initial infection	0	0	0		4+	4+	4+	
	12 days after reinoculation	0	0	0		0	0	0	
1897	Normal	4+	4+	4+		4+	4+	4+	
	13 days after initial infection	0	0	0		4+	4+	4+	
	12 days after reinoculation	0	0	0		1+	1+	0	
1809	Normal (not obtained)								
	12 days after initial infection	0	0	0	0	4+	4+	4+	4+
	11 days after reinoculation	0	0	0	0	0	0	0	0

* 0 = mouse with no pulmonary lesions at autopsy. 1+ to 4+ = mice with progressive degrees of influenzal pneumonia; 4+ indicates a complete and fatal pneumonia.

event, even though human virus did penetrate the respiratory tract mucosa, it would be incapable of eliciting a specific antibody response. The following experiments were conducted in an attempt to determine the applicability of the second hypothesis.

Five swine were infected in the usual way with swine influenza. After recovery they were reinoculated with human influenza virus, but, instead of administering the virus intranasally as in the experiments outlined in Table III, it was given intramuscularly. The animals exhibited no evidence of illness and after a period of observation of 11 or 12 days were bled. Serum obtained at this time, together with that secured before and after the swine influenza infections, was tested for the presence of neutralizing antibodies for the swine and human influenza viruses. The results of these neutralization experiments are given in Table IV.

As shown in Table IV, 3 of the 5 swine influenza-immune swine, inoculated intramuscularly with human influenza virus, developed antibodies which completely neutralized the human virus; 1 animal, swine 1897, developed antibodies which neutralized partially; while the 5th animal, swine 1893, neutralized the human virus partially both before and after its intramuscular injection.

These experiments indicate that the usual failure of intranasally administered human influenza virus to elicit specific neutralizing antibodies in swine influenza-recovered swine is not due to interference with or exhaustion of the mechanism responsible for antibody elaboration.

DISCUSSION

It has been found that swine recovered from infection with swine influenza or swine influenza virus alone are usually immune to infection with a mixture of human influenza virus and *H. influenzae suis*, and that they rather promptly render human virus, administered intranasally, non-demonstrable. This cross-immunity is not associated with the presence of demonstrable neutralizing antibodies for the human virus in the sera of the immune animals. Furthermore, antibodies for the human virus usually fail to develop even after reinoculation intranasally with that agent. Swine immune to human influenza infection, by virtue of a previous attack of swine influenza, thus behave towards the human virus much like naturally refractory animals in that they are resistant to infection without possessing virus-neutralizing antibodies, they do not permit the establishment in the respiratory tract of virus given intranasally, and they usually fail to develop virus-neutralizing antibodies following intranasal inoculation.

Antibodies against human influenza virus do appear, however, in

the sera of swine influenza-immune swine to which the human virus is given intramuscularly. This indicates that their failure to appear after intranasal inoculation is not due to interference, by previous swine virus infection, with the mechanism responsible for antibody elaboration. Rather it suggests that the failure may have resulted from inability of the virus to penetrate the respiratory tract mucosa deeply enough to produce an antibody response. It seems likely that, in swine, the cross-immunity to human influenza virus established by previous infection with swine influenza virus is the result of an acquired barrier to the entrance of human virus into the respiratory tract mucosa.

The cross-immunity conferred against swine influenza by the human influenza virus differs from that in the reverse direction just discussed, and here the association of *H. influenzae suis* in the initial infection is important. Swine recovered from infection with a mixture of human influenza virus and *H. influenzae suis* are usually immune to swine influenza, while those whose initial infections have been with the human virus alone are usually still susceptible to swine influenza, although they develop milder attacks than the control animals. Furthermore, while the pneumonias exhibited by these non-immune swine at autopsy are qualitatively similar to those seen in swine influenza in fully susceptible animals, swine influenza virus is either not demonstrable or is present only in low concentration in the turbinates and lungs. This finding is in striking contrast to the uniformity with which virus is demonstrable in the lungs and turbinates of the control swine.

Antibodies capable of neutralizing swine influenza virus are not present in the sera of animals recovered from human influenza, but they do appear in the sera of most such swine following reinoculation with swine influenza, and this even in the absence of clinical manifestations of infection. The finding indicates that the immunity to swine virus conferred by previous infection with the human agent is not of such a nature as to give rise to a barrier to virus invasion in the respiratory tract mucosa of the apparently immune host.

The fact that the respiratory tract mucosas of swine still let swine influenza virus through after recovery from infection with the human virus may explain why infection with a mixture of human virus and

bacterium gives a better immunity to swine influenza than does infection with human virus alone. Swine initially infected with a mixture of human influenza virus and *H. influenzae suis* develop an immunity to both agents: immunity to the human virus is evidenced by the appearance of specific neutralizing antibodies, while immunity to *H. influenzae suis* is indicated by the failure of this bacterium to become established in the lower respiratory tract upon reinoculation with swine influenza. Swine initially infected with human influenza virus alone, on the other hand, become immune only to this virus. When later inoculated intranasally with a mixture of swine influenza virus and *H. influenzae suis* the animals immune to both the human virus and *H. influenzae suis* have only the heterologous virus with which to deal. The swine virus in these cases, to judge by the formation of swine virus-neutralizing antibodies, invades the tissues of the respiratory tract and persists for a short time at least. That it is rather promptly inactivated, however, probably through an immunity mechanism established as a result of previous infection with the closely related human virus, is indicated by the fact that, in animals that remain free of symptoms, no swine virus can be demonstrated in the turbinates or lung even 3 days after inoculation. The swine show no clinical or postmortem evidence of this evanescent virus infection and thus, like ferrets and mice, appear to possess a perfect cross-immunity. On the other hand, swine immune only to the human virus cannot usually adequately resist this transitory infection with the swine influenza virus when a concomitant *H. influenzae suis* infection is added. Even here, however, the virus component is rapidly destroyed in the influenzal lesions it has initiated, as evidenced by its complete absence, or presence only in low concentration, in the turbinates and lungs as early as the 3rd day after infection.

To judge from the two instances in which swine were given 2 intranasal injections of human influenza virus alone, repeated inoculations with the human virus enhance the effectiveness of the cross-immunity defense mechanism against swine influenza.

It seems likely, from the experiments discussed, that the cross-immunity shown by swine recovered from infection with the viruses of human and swine influenza, respectively, may be due to different mechanisms. Animals convalescent from swine influenza are immune

to human influenza virus apparently by virtue of the failure of the human agent to get through the lining of the respiratory tract.   In the case of swine recovered from infection with human influenza virus, on the other hand, the respiratory tract mucosa still lets the swine influenza virus pass, but here the invading virus is rather promptly inactivated by some unknown defense mechanism evidently established by the earlier human virus infection.

The findings recorded were all obtained in "acute" experiments and it is possible that other results would be obtained when long periods of time intervened between succeeding exposures to infection.   Practical considerations, incident to experimental work with swine, have made it impossible to include such long time experiments in the present studies.

SUMMARY

Swine recovered from infection with either swine influenza or swine influenza virus alone are usually not only immune but refractory to human influenza infection.   Swine recovered from infection with a mixture of human influenza virus and *H. influenzae suis* are usually immune to swine influenza while those recovered from infection with human influenza virus alone are usually not immune to swine influenza. The possible mechanisms involved in the cross-immunity between the influenza viruses are discussed.

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THE EFFECT OF HEMOPHILUS INFLUENZAE SUIS VACCINES ON SWINE INFLUENZA

By RICHARD E. SHOPE, M.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

Early experiments indicated that the bacterium, *Hemophilus influenzae suis*, (1) administered intranasally to swine, conferred no immunity to swine influenza (2). These had not been intended to test the possible value of the organism for use as a prophylactic agent in controlling swine influenza, and no experiments in which the bacterium was administered in other ways than intranasally were conducted. More recent experiments (3), however, have indicated that, when accompanied by human influenza virus, *H. influenzae suis* does play a rôle in immunizing swine to swine influenza. It has been found that while swine recovered from infection with a mixture of human influenza virus and *H. influenzae suis* were usually immune to swine influenza, those recovered from infection with human influenza virus alone were usually not immune. These experiments were believed to indicate that *H. influenzae suis*, in the presence of a concomitant human influenza virus infection, immunized swine to the bacterial component of the swine influenza etiological complex (4).

The apparent discrepancy between the earlier and the more recent experiments could be explained by assuming that when the bacterium alone was administered intranasally to swine it was applied superficially to an intact mucosa that was impermeable to its deep penetration. The inability of the bacterium to penetrate the respiratory tract mucous membranes could account for its failure to induce an immunity response. However, when *H. influenzae suis* was given intranasally to swine in mixture with human influenza virus it was not only afforded a portal of entry into deeper tissues through lesions produced by the virus, but the influence of the virus may have en-

dowed it with invasive properties that it did not possess alone. Under such circumstances the bacterium might be expected to elicit an immunity response. This possible explanation of the observed phenomena raised the question of whether *H. influenzae suis* vaccines given intramuscularly might not immunize swine to the bacterial component of the etiological complex of swine influenza.

EXPERIMENTAL

Preparation of H. influenzae suis Vaccines.—Cultures 18 and 23 *H. influenzae suis*, originally obtained from naturally occurring field cases of swine influenza, were used either singly or pooled in the experiments. The 48 hour growths from chocolate agar slants were scraped off and suspended in a small amount of physiological saline. These suspensions were then centrifuged in graduated tubes for ½ hour at 1600 to 1800 R.P.M. The volume of bacterial sediment was noted after which the sediment was resuspended in sufficient physiological saline to make a final 1 per cent by volume suspension. Part of the suspension was removed to use as living vaccine while the remainder was heated at 57°C. for 30 minutes in sealed tubes submerged in a water bath. All heated suspensions proved sterile when planted on media capable of supporting the growth of *H. influenzae suis*.

At the time that the present experiments were conducted no recently isolated strains of *H. influenzae suis* were at hand. The two strains used had both been under cultivation sufficiently long that, while still capable of producing influenza when given intranasally to swine in mixture with swine influenza virus, they no longer transferred with the virus from sick to normal animals by contact. The ability of the bacterium to transfer together with the virus from swine to swine by pen contact is a property possessed by all freshly isolated cultures of *H. influenzae suis* which is lost after a variable period of cultivation on artificial media (5). The experiments to be reported were conducted with non-contagious strains of the bacterium.

Vaccination of Swine with Heated and Living Hemophilus influenzae suis

Each of 8 swine were given 3 intramuscular injections at 8 day intervals of heat-killed *H. influenzae suis*; a second group of 6 swine received injections similarly of living *H. influenzae suis*. The amount of the first dose administered was 1 cc., while the 2 succeeding doses were of 2 cc. each. The heated vaccine caused no apparent reaction in any of the animals. The living vaccine, however, caused a sharp temperature elevation on the day following the second injection.

The vaccinated animals were tested for immunity to swine influenza, 9 to 14 days after their last dose of vaccine, by intranasal inoculation with a mixture of

swine influenza virus and *H. influenzae suis*. After either 3 or 4 days of clinical observation they were killed and autopsied and their respiratory tracts examined for lesions of influenza. Details of the experiments and the outcome of the tests for immunity are given in Table I.

As shown in the table, the results obtained were not clear cut and there was considerable individual variation in the degree of protection afforded. In only one instance, that of swine 1690, was protection against the effects of *H. influenzae suis* apparently complete. The disease seen in this animal was typical, both clinically and at autopsy, of that produced by the virus alone (4); and *H. influenzae suis* could not be cultivated from the respiratory tract. The remaining 7 swine, vaccinated with heated *H. influenzae suis*, developed, when tested for immunity, a swine influenza that was less severe clinically than that shown by any of the 3 control swine. At autopsy, the influenzal pneumonia encountered in the vaccinated pigs was found to involve from 1.5 to 3.5 lobes, whereas, in the control animals, 4 and 5 lobes were consolidated. Virus, demonstrable by mouse inoculation (6), was present in the lungs of all of the pigs, but *H. influenzae suis* could not be grown from the affected lungs of 3 of the 8 vaccinated animals, although it was present higher in the respiratory tract in 2 of the 3 cases. It seems likely that the suppression of *H. influenzae suis* in these 3 swine was an effect of the immunization procedure.

The swine vaccinated with living *H. influenzae suis* differed somewhat from those that had received heated vaccine. When tested for immunity to swine influenza they became severely ill within 24 hours, lay prostrate, and exhibited temperatures of 41°C. or higher. The control swine at this time were only slightly ill, and showed less elevation of temperature. On the 2nd day, however, the vaccinated animals were much improved and no case at this time could have been classified clinically as more than a mild swine influenza. Their temperatures dropped either to normal or to low fever level and remained there. The control swine, on the other hand, became progressively worse and exhibited the signs of typical swine influenza. At autopsy the difference in the extent of pneumonia shown by the vaccinated pigs and the control animals was not striking, and probably in the cases of the last 4 animals in Table I it was negligible. However, the amount of consolidation in the lung of neither control animal was as

TABLE I

Effect of Hemophilus influenzae suis Vaccines on Swine Influenza

Swine No.	Vaccination — Three intramuscular inoculations with	Result	Time between vaccination and immunity test	Test for immunity to swine influenza* — Clinical illness	Findings in lungs at autopsy — Extent of lesions	Virus	H. influenzae suis
			days				
1663	Heat-killed H. influenzae suis	No illness	9	Mild influenza	2†	Present	Absent
1667	" " "	"	9	2 day fever; not definitely ill	1.5	"	Present
1690	" " "	"	9	"Filtrate disease"	0.3	"	Absent
1693	Nil, control for above 3 vaccinated swine			Typical swine influenza	4.3	"	Present
1708	Heat-killed H. influenzae suis	No illness	14	Mild influenza	3.5	"	"
1709	" " "	"	14	4 day fever; mildly ill	3	"	"
1710	" " "	"	14	4 " " " "	2.5	"	"
1712	" " "	"	14	4 " " " "	2	"	Absent
1713	" " "	"	14	4 " " " "	3	"	Present
1716	Nil, control for above 5 vaccinated swine	"		Typical swine influenza	5	"	"
1725	" " " 5 "	"		" "	5.3	"	"
1731	Living H. influenzae suis	Fever 1 day after 2nd injection	10	Fever and prostration 1st day; mildly ill later	1.5	"	Absent
1735	" " "	"	10	4 " " " "	1.5	"	"
1737	" " "	"	10	4 - " " "	1.5	"	"
1732	Nil, control for above 3 vaccinated swine			Severe swine influenza	2.5 (bilateral pleuritis)	"	Present

1730	Living *H. influenzae suis*	Fever 1 day after 2nd injection	9	Fever and prostration 1st day; mildly ill later	2.3	"	Absent
1743	" " "	" "	9	" "	2.3	"	Present
1745	" " "	" "	9	" "	1.7	"	Absent
1754	*Nil, control for above 3 vaccinated swine*	Typical swine influenza			2.3	"	Present

* Intranasal inoculation with mixture of swine influenza virus and *H. influenzae suis*. (The swine lung has 7 lobes.)

† Pneumonia expressed in number of lobes involved.

extensive as is usual in typical swine influenza, although one had a bilateral fibrinous pleuritis. Virus was present in the lungs of all pigs, but *H. influenzae suis* could be grown from the pneumonic lung of only 1 of the 6 vaccinated animals despite its presence higher in the respiratory tracts of all. As in the case of the animals that received heated vaccine, this suppression of *H. influenzae suis* in the lung is considered an effect of the immunization procedure.

None of the sera of the vaccinated swine, drawn just prior to their test for immunity, exerted any neutralizing effect on the swine influenza virus. Neither did they contain agglutinins for *H. influenzae suis*.

DISCUSSION

The results obtained in the present experiments, when considered as a whole, furnish evidence that *H. influenzae suis* given intramuscularly to swine elicits an immune response capable of modifying the course of a later swine influenza infection. Heated vaccine appears to be at least as effective as a living one so far as can be judged from clinical and postmortem findings. However, *H. influenzae suis* was more often completely suppressed in the pneumonic lungs of animals vaccinated with live vaccine than in the lungs of those that had received the heated vaccine. If this suppression of the specific bacterium is really an effect of the immunization procedure, then more protection was achieved by the living vaccine. The severe clinical reaction, with extreme prostration and high fever, occurring within 24 hours of the test for immunity in the swine vaccinated with living organisms, may represent an allergic reaction in which destruction of *H. influenzae suis* occurs in the lung. Certainly the prompt clinical improvement shown by these animals after their initial reaction suggests that the swine influenzas they suffer are not progressive after the first 24 hours, and that the factors responsible for the continued illness of the control swine are no longer operative in them. Their condition corresponds to that seen at the onset of convalescence on the 5th or 6th day postinfection in susceptible swine when, though still carrying anatomical changes caused by influenza, they appear clinically almost normal. In the animals treated with heated vaccine, on the other hand, suppression or destruction of *H. influenzae suis* in the respiratory tracts

appears to be less drastic and, while partial protection is evident from both clinical and postmortem examination, the immediate severe reaction following the test for immunity is avoided. No explanation for this difference in the character of immunity established by heated and living *H. influenzae suis* vaccines is apparent. Agglutinins for *H. influenzae suis* were not demonstrable in the sera of any of the vaccinated swine at the time of the test for immunity to swine influenza.

From the practical standpoint of controlling swine influenza the partial protection afforded swine by the bacterial vaccines is of no immediate value since it is already known that complete protection to the disease can be achieved by means of swine influenza virus vaccines (2, 7). The present studies are of interest only in showing that at least a partial immunity to the bacterial component of the etiological complex responsible for swine influenza can be established, and that this is capable of modifying the course of a later swine influenza infection. Swine influenza virus vaccines remain the method of choice in immunizing swine to swine influenza.

SUMMARY

Either living or heat-killed *H. influenzae suis* vaccines, given intramuscularly to swine, elicit an immune response capable of modifying the course of a later swine influenza infection. The protection afforded is only partial and is in no way comparable to the complete immunity afforded by swine influenza virus vaccines.

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DEMONSTRATION OF PASSIVE IMMUNITY IN EXPERIMENTAL MONKEY MALARIA

By L. T. COGGESHALL, M.D., and H. W. KUMM, M.D.

(From the Laboratories of the International Health Division, The Rockefeller
Foundation, New York)

It is generally accepted that malaria infections confer a certain
degree of immunity on their hosts upon recovery. It has not been
clearly shown, however, that the serum of an animal after recovery
from the acute attack contains immune substances which are capable
of exerting a protective action when injected into a normal animal.

In the literature there are conflicting reports concerning the protective property
of serum taken from animals or man suffering from chronic malaria. Taliaferro
and Taliaferro (1), working with canaries, and Nauck and Malamos (2), using
monkeys, reported their failure to produce passive immunity with malaria immune
serum, irrespective as to whether it was given before, at the time, or after inocula-
tion of the parasite. However, Findlay and Brown (3), working with canaries,
believed that they could demonstrate some protective action if they used suffi-
ciently large doses of immune serum in conjunction with a very small infective
dose of parasites. In induced human malaria Kauders (4) felt that convalescent
serum had some protective action as well as a beneficial therapeutic effect. Soti-
riadès (5) reported clinical improvement in seven malaria-infected paretics after
intramuscular injections of 20 cc. of whole blood taken from individuals with
chronic malaria; in one case the injections of serum were combined with adminis-
tration of small amounts of quinine.

When *Plasmodium knowlesi* is injected into *Macacus rhesus* the resultant in-
fection almost invariably terminates in death. Mulligan and Sinton (6) reported
that in a series of over 120 monkeys infected with this parasite only one monkey
recovered spontaneously from the initial attack. In our experience there was
only one survival among 70 infections. An infection with this parasite, however,
may be converted into a chronic state by administering antimalarial drugs early
in the course of the disease.

Materials and Methods

Two species of monkey plasmodia were represented in this series of experiments.
One was a strain of *Plasmodium knowlesi* (Sinton and Mulligan) obtained through

the courtesy of Sir S. Rickard Christophers at the London School of Hygiene and Tropical Medicine in 1934. The other was a strain of *Plasmodium inui* (Halberstadter and Prowazek) isolated in this laboratory in 1933 from a naturally infected *Macacus cynomolgus*. *Macacus rhesus* monkeys were used exclusively in these experiments. They were infected by intravenous injection of citrated blood containing a known number of parasites.

The so called immune serum used in the protection tests described below was collected and pooled from animals with chronic infections of varying duration. The time interval between quinine treatment and bleeding varied from 15 to 190 days, or an average of 41 days. In the pool there was included serum of only one monkey which had been given atabrine instead of quinine. This animal had received one dose of the drug and it was not bled until 28 days after treatment. All bleedings were done under ether anesthesia.

In the early experiments the donor animals were exsanguinated. Later, however, a more economical procedure was adopted by bleeding the animals at infrequent intervals without complete exsanguination. It was found that an amount up to 50 cc. of blood could be obtained without sacrificing the animal. Red blood cell counts were made on all monkeys which had been bled and it was found possible to repeat the bleedings at intervals of approximately 3 weeks. The injections of serum were generally given intraperitoneally, with the exception of one experiment in which some of the serum was injected by the intramuscular route.

After injection of the parasites, blood examinations were made daily on all animals to acquire information concerning the progress and intensity of the infection. The number of parasites found in the blood of infected monkeys, as shown in Table I, is based upon the number of parasitized cells per 10,000 normal red blood corpuscles. In Charts 1 to 5 inclusive the *P. knowlesi* counts were plotted on an arithmetical scale with the ordinates compressed and in Chart 6 the *P. inui* counts were plotted on an unmodified arithmetical scale. When estimating the number of parasites to be used for an inoculation, a simultaneous red cell and parasite count was made on the donor animal. From the data thus obtained the volume of infected blood necessary to contain the desired number of parasites was computed.

EXPERIMENTAL

Experiment 1.—A normal *rhesus* monkey, No. 1, was given 22 cc. of pooled serum intraperitoneally from animals with chronic infection. 3 hours later this animal and another normal monkey, No. 2, which served as control, were each given 1,600,000 parasites intravenously. The course of infection in these two animals is shown in Chart 1 and Table I. It will be noted that the control monkey died on the 8th day after inoculation, while in monkey 1 the course was considerably prolonged and the animal did not succumb to the infection until the 13th day.

Experiment 2.—In this experiment a normal monkey, No. 3, was given 15 cc. of pooled serum from monkeys having chronic infection. Another animal, No. 4,

received a similar amount of normal monkey serum to serve as a control. 3 hours later these two animals and also an additional monkey, No. 5, were each inoculated with 4,500,000 parasites. Immediately following the injection of parasites monkey 3 was given mn additional 15 cc. of pooled serum from monkeys with chronic infection, and similar amounts were injected daily for the following 4 days. Monkey 4 received equivalent amounts of normal serum at the same time intervals. The results of this experiment nre shown in Chart 2 nnd Table I. It will be seen that monkey 3, which received pooled serum from monkeys with chronic infection, survived. Monkey 4, which was given normal serum, died on the 8th day, and monkey 5, which received no serum, died on the 12th day following inoculation.
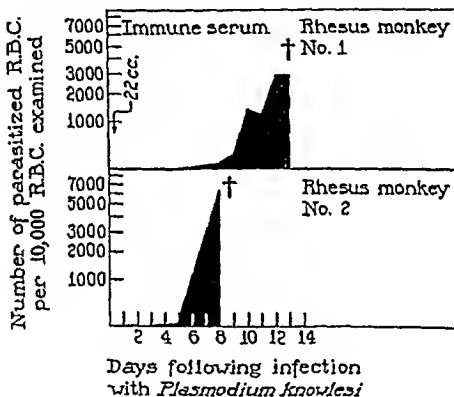
CHART 1

Experiment 3.—The foregoing experiment was repeated along similar lines, except that a different pool of immune serum was used and a normal serum control was not included. Two normal monkeys, Nos. 6 and 7, were each inoculated with 6,000,000 parasites. Monkey 6 had received 25 cc. of immune serum 3 hours prior to the injection of parasites and was given additional injections of immune serum at intervals, as used for monkey 3 in the preceding experiment, and it survived (Chart 3 and Table I). The control animal, No. 7, died on the 7th day after inoculation with the parasites.

Since it was evident from Experiments 2 and 3 that the serum from monkeys harboring chronic malarial infections is capable of conveying

Summary of Protection Test Results in Monkeys, Showing the Amount of Immune Serum Inje[cted]
Daily Parasite Count in the [...]

Serial No. of monkey	No. of parasites injected	3 hrs. before Amount serum	3 hrs. Parasite count	Simultaneous Amount serum	Simult. Parasite count	1 Amount serum	1 Parasite count	2 Amount serum	2 Parasite count	3 Amount serum	3 Parasite count	4 Amount serum	4 Parasite count	5 Amount serum	5 Parasite count	6 Amount serum	6 Parasite count
		cc.		cc.		cc.		cc.		cc.		cc.		cc.		cc.	
1	1,600,000	22					0		0		0		0		3		3
2 c	1,600,000						0		0		4		4		29		790
3	4,500,000	15		15		15	0	15	0	15	0	18	0		0		1
4 C	4,500,000	15		15		15	0	15	0	15	0	15	2		120		366
5 c	4,500,000						0		0		1		5		43		330
6	6,000,000	25		15		12	0	17	0	16	1	24	2		1		3
7 c	6,000,000						0		0		10		111		784		952
8	500,000,000			25			0	5	1	5	6	5	4	5	11	5	14
9 c	500,000,000						6		32		226		400		1440		5732
10	1,000,000			5		5	0	5	0	5	0	5	0		0		5
11	1,000,000			10		10	0	10	0	10	0	10	0		0		0
12 c	1,000,000						0		0		0		0		0		0
13	3,370,000			5		5	0	5	0	5	5	5	24	5	33	5	836
14	3,370,000			5		5	0	5	1	5	5	5	6	5	136	5	278
15	3,370,000			5		5	0	5	1	5	1	5	4	5	47	5	24
16	3,370,000			5		5	0	5	1	5	2	5	4	5	37	5	6
17	3,370,000						0		0		2		4	5	74	5	44
18	3,370,000						0		0		1		5	5	5	5	123
19 C	3,370,000			5		5	0	5	0	5	1	5	1	5	6	5	18
20 C	3,370,000			5		5	1	5	0	5	2	5	75	5	96	5	1005
21	1,407,000			5		5	0	5	0	5	0	5	0	5	0	5	1
22	1,407,000			5		5	0	5	0	5	0	5	0	5	0	5	2
23	1,407,000			5		5	0	5	0	5	0	5	0	5	1	5	9
24	1,407,000			5		5	0	5	0	5	0	5	0	5	1	5	1
25	1,407,000						0		0		7	5	17	5	112	5	612
26 C	1,407,000			5		5	0	5	0	5	0	5	25	5	115	5	654
27 C	1,407,000			5		5	0	5	0	5	4	5	10	5	84	5	25

D = died.   S = survived.   C = control, received normal monkey serum. c = control, r[eceived]...

I

the [...] Intervals Following Inoculation with the Malaria Parasites (Plasmodium knowlesi) and also the Daily [...] Blood of the Infected Animals

			8	9		10		11		12		13		14		15		16		17		18		19	
Parasite count	Amount serum	Parasite count	Parasite count	Amount serum	Parasite count	Amount serum	Parasite count	Amount serum	Parasite count	Amount serum	Parasite count	Amount serum	Parasite count	Amount serum	Parasite count	Amount serum	Parasite count	Amount serum	Parasite count	Amount serum	Parasite count	Amount serum	Parasite count	Amount serum	Parasite count	
			cc.		cc.		cc.		cc.		cc.		cc.		cc.		cc.		cc.		cc.		cc.		cc.	
0	0		81	203		1300		1160		2840		D														
4	4		6186	D																						
0	0		24	6		32		10		162		51		49		84		51		148		12		7	S
0	5		6840	D																						
			1460	3354		1648		3156		D																
2			65	63		308		360		750		349		154		25		7		2		—		195	S
			D																						
			331	844		651		1174		1848		962		1767		6039		D							
5	5		2	46		190		184	5	470	5	1098	3	72		218		140		370		165		96	S
5			13	73		89		368	10	1152	10	504	5	145		80		48		159		53		91	S
5			1	1		7		93	10	125	10	299		778		804		887		2985		4716		1710	D
			2645	5	5215	5	D																		
5	5		1166	5	932	5	4036																		
5	5		210	396		435		333		152		97		68		64		69		16		6		0	S
5	5		32	32		41		41		103		32		95		32		29		78		95		6	S
5	5		290	5	139	5	100	5	93		32		2		1		14		76		34		52		28	S
2			366	5	804	5	1449		D																
2			120	5	242	5	467	5	932	5	2056		524	5	3780		5130		D						
1			D																						
1	5		12	5	20	5	174	5	235	5	172	5	756		1402		D								
2			11	5	6	5	74	5	97	5	366	5	740		2805		6552		D						
0			45	10	637	10	1802	10	3702		D														
0			23	10	103	10	308	10	2542	10	1968	10	3575		D										
0			6812	D																					
0			D																						
7			D																						

I monkey serum.   c = cc.   no serum.

CHART 2

passive immunity to normal animals, it was decided to subject the protective property of the serum to a more severe test.

Experiment 4.—Two normal monkeys, Nos. 8 and 9, were each inoculated with 500,000,000 parasites, which was approximately 100 times the number used in the previous experiments. Monkey 8 had received 25 cc. of immune serum 3 hours prior to the injection of the parasites and 5 cc. daily for 5 days thereafter. The results are shown in Chart 4 and Table I. It will be noted that in spite of inoculation with an overwhelmingly large number of parasites and the use of a relatively small amount of immune serum, monkey 8 had a prolonged course of infection and
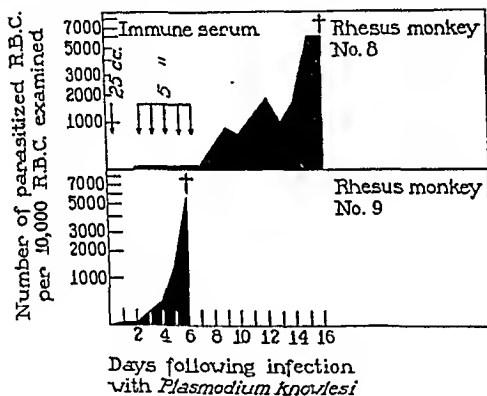
CHART 4

did not die until the 16th day of the disease, whereas the control animal, No. 9, died on the 6th day after inoculation.

An experiment was now undertaken to determine the protective property of varying amounts of immune serum against a relatively small infecting dose of the parasites.

Experiment 5.—Three normal monkeys, Nos. 10, 11, and 12, were each inoculated with approximately 1,000,000 parasites. Monkey 10 received 5 cc. of immune serum daily for 5 days, and monkey 11 was given daily injections of 10 cc. for a similar period, while monkey 12 served as control. It is frequently observed that when the infecting dose of the parasites is small, the incubation period is

considerably prolonged. In this experiment the control monkey, No. 12, did not die until the 19th day after inoculation. After a prolonged incubation period monkeys 10 and 11 showed an intensity of infection similar to the control animal. However, when additional injections of immune serum were given at the periods as indicated in Chart 5 and Table I, there was a rapid fall in the number of para‑sites present in the circulating blood and both animals recovered.
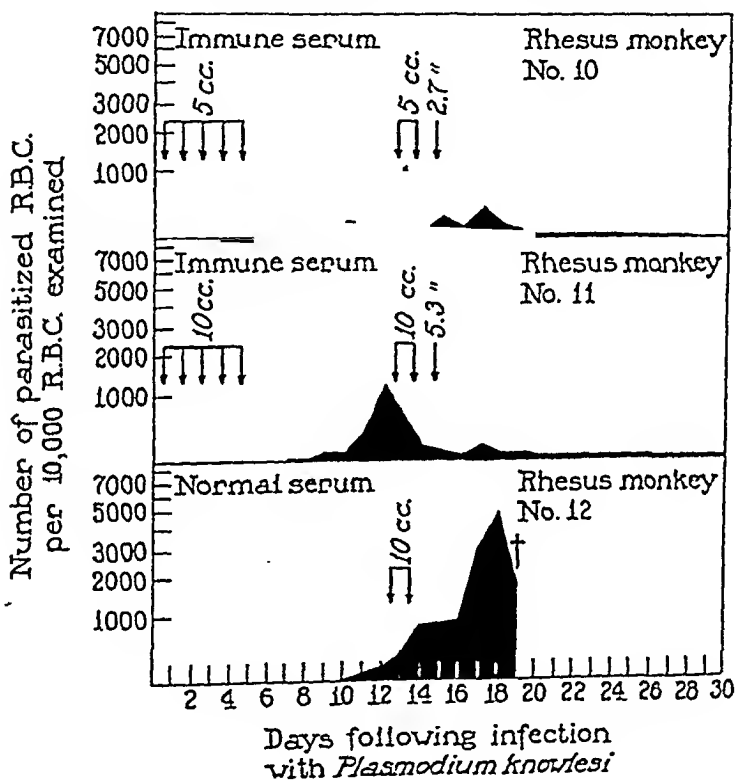
CHART 5

With a view to determining the most favorable time for the adminis‑tration of immune serum in order to afford maximum protection, the following experiment was carried out.

Experiment 6.—Eight normal monkeys, Nos. 13 to 20 inclusive, were each inoculated with 3,370,000 parasites. As shown in Table I, monkeys 13 and 14 were given 5 cc. of immune serum daily during the entire course of the disease;

Nos. 15 and 16 each received 5 cc. daily during the first 7 days; Nos. 17 and 18 were given no serum until the infection had become well established, which was
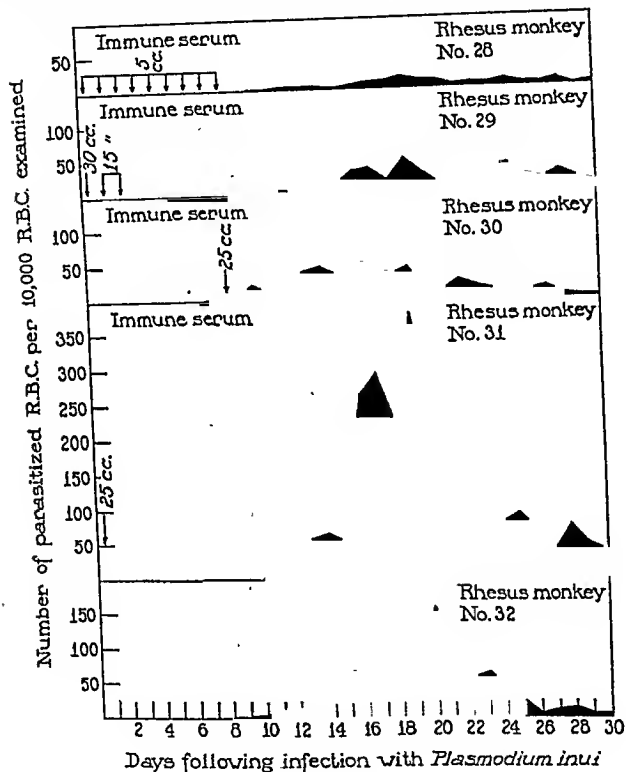
CHART 6

on the 5th day, and then were given 5 cc. daily for the duration of the disease. The control monkeys, Nos. 19 and 20, each received 5 cc. of normal monkey serum

daily throughout the course of their infection. As seen in Table I, monkeys 13 and 14 died of acute malaria on the 10th and 11th day, respectively; Nos. 15, 16, and 17 survived, while No. 18 also died on the 10th day after inoculation. The control animals, Nos. 19 and 20, died on the 16th and 8th day, respectively.

The results seemed to indicate that while the immune serum afforded good protection in some animals, it completely failed in others. We have no adequate explanation for this phenomenon, but it is suggested that besides individual variation in the susceptibility of monkeys, there may be a considerable variability in the concentration of the immune substances in the serum of individual monkeys harboring chronic infection from which the pools were made up.

The preceding experiment was repeated except that a different pool of immune serum was used and the infecting inoculum contained a smaller number of parasites.

Experiment 7.—Seven normal monkeys, Nos. 21 to 27 inclusive, were each inoculated with 1,407,000 parasites and the immune serum was given at the same intervals as in Experiment 6. The results are shown in Table I. Monkeys 21 and 22, which received 5 cc. of serum daily throughout the course of infection, died on the 15th and 16th day, respectively, after injection of the parasites; monkeys 23 and 24, each of which was given 5 cc. of immune serum daily during the first 7 days of infection, died on the 12th and 14th day, respectively. Monkey 25, which received 5 cc. of immune serum daily, commencing after the infection was well established, died on the 8th day. The two control animals, Nos. 26 and 27, both died on the 7th day after inoculation.

It seemed obvious from these results that the immune pool used in this experiment had only a slight protective property, as shown by the prolongation of the course of infection in monkeys which received daily injections of 5 cc. each throughout the disease. This serum appeared to have been ineffective when given after the infection had already become firmly established.

All the foregoing experiments were carried out with *P. knowlesi.* In order to determine whether a chronic infection with another species of malaria parasite results in the production of protective substances in the serum of the host, the experiment described below was carried out with *P. inui.* When injected into *rhesus* monkeys *P. inui* generally produces only a moderately severe infection which practically always is followed by spontaneous recovery. A number of monkeys

which had recovered from an initial infection with this parasite were bled and the sera pooled. The protective effect of this serum against *P. inui* infection was determined by the intensity of infection as shown by daily counts of parasitized cells per 10,000 normal red cells.

Experiment 8.—Five normal monkeys, Nos. 28 to 32 inclusive, were each inoculated with 2,500,000 parasites. The results of this experiment are shown in Chart 6. Monkey 28, which received 5 cc. of serum for 9 consecutive days after the administration of parasites, had a maximum count of 16 parasites per 10,000 normal red blood cells on the 19th day of infection. Monkey 29, which received 15 cc. of serum 2½ hours before inoculation with parasites, 15 cc. at the time of infection, and 15 cc. daily for 2 days, had a maximum count of 59 on the 23rd day of the disease. Monkey 30 received 25 cc. of serum on the 8th day after the onset of infection and none thereafter; the maximum count was 56 on the 16th day. Monkey 31 received only one serum injection of 25 cc. This was administered 2½ hours before inoculation with parasites and the maximum parasite count was 380 on the 19th day. Monkey 32, the control, received no serum and had a maximum count of 160 which occurred on the 20th day of the infection.

It is to be noted in this experiment that serum from animals which had recovered from the acute attack due to *P. inui* was most effective in decreasing the intensity of the infection when administered during the course of the disease.

DISCUSSION

There are numerous reports in the literature which suggest the formation of specific antibodies in the host during the course of an infection due to malaria parasites. In avian malaria, for example, the recovery from an initial attack occurs with such rapidity that it is often referred to as a crisis. Since it is known that from the onset of the infection the macrophages of an infected bird are constantly phagocytizing parasites, the marked decrease in their number at the time of recovery could probably best be explained on the basis of opsonizing antibodies or humoral immune substances. Furthermore, the fact that an animal after recovery from a malarial infection is usually immune to reinfection with the homologous parasite would tend to suggest that there are specific protective substances present in the serum of such an animal. These observations have led Taliaferro and Cannon (7), Ciuca (8, 9), Hackett (10), Ferrio (11), Thomson (12), Neumann (13), and others, to infer that specific humoral immune

substances to malaria parasites are present in the serum of the host during chronic infection. But it is usually pointed out that these antibodies appear in the serum in such a low concentration that their presence cannot be demonstrated by ordinary protection test methods.

We believe that the data presented in this paper offer definite evidence to indicate that, in some instances at least, protective substances do occur in the serum of monkeys harboring chronic infection with *P. knowlesi* and *P. inui*, and that the serum of such animals, when injected into normal monkeys, is capable of conveying passive immunity in the animal recipient to the homologous parasites. Our results also indicate that the protective antibodies appear in the serum of animals with chronic infection in a very low concentration, and that large amounts of the serum must be used in order to demonstrate their presence. In our experiments amounts varying from 22 to 109 cc. were given to an animal, which makes an average of 52 cc. per animal, or 26 cc. per kilo of body weight. Pooled serum from a number of monkeys was used in these experiments, and as shown by the results there was a considerable variation in the protective property of different pools. This would seem to suggest that a wide variation probably exists in the degree of humoral immunity in monkeys suffering from chronic malaria.

Our results suggest that the protective action of the immune serum was most pronounced when the serum was administered in daily doses throughout the course of the experimental disease. Relatively large amounts given shortly before or at the time of the injection of the parasites seemed to have only a minor influence on the course of infection. Likewise, when given after the infection was already well established the serum seemed to have little effect on the final outcome. Of special interest are monkeys 10 and 11, in Experiment 5, which received immune serum both early and late in their infection. As seen in Table I, after the first course of serum treatment had ceased, the parasite count in the blood of these two monkeys rose to 1098 and 1152, respectively. Immune serum given at this point resulted in a fall in the parasite count to 72 and 504, respectively, within 24 hours. It has often been observed that when the parasite count has reached 1000 in an initial attack of *P. knowlesi* infection it is impossible to prevent death even with massive doses of quinine.

In preparing the immune serum pools used in the experiments described above, a considerable number of the monkeys with chronic malarial infection were bled repeatedly and some as many as six times. Red blood cell counts done at frequent intervals on these animals showed that it required about 3 weeks after each bleeding before the normal counts were restored. After bleeding these monkeys frequently showed temporary macrocytosis and polychromasia. Parasite counts usually showed little or no alteration after the multiple bleedings. One animal, however, suffered a relapse and died of acute malaria in spite of massive quinine therapy. Following successive bleedings of the original monkeys with chronic infection, the potency of the pooled immune serum seemed to decrease progressively as evidenced by its ability to protect normal animals.

SUMMARY AND CONCLUSION

A *Plasmodium knowlesi* infection in *rhesus* monkeys is almost invariably fatal. This infection, however, may be made chronic by the early administration of antimalarial drugs. The animals then will harbor a chronic infection for an indefinite period. The serum taken from monkeys with chronic infection and injected into those suffering from an acute attack was found to have a definite depressing effect upon the course of the experimental disease. In some instances death was prevented and the acute infection changed into a chronic form; in others, the course of the experimental disease was prolonged.

In a similar manner the serum from monkeys harboring a chronic *Plasmodium inui* infection, when injected into monkeys suffering from an acute attack due to this parasite, was found to be effective in reducing the intensity of the primary infection.

The data presented indicate that protective antibodies are produced in the serum of monkeys during experimental malaria infection.

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CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

XI. THE SPECIFICITY OF AZOPROTEIN ANTIGENS CONTAINING GLUCURONIC AND GALACTURONIC ACIDS

BY WALTHER F. GOEBEL, PH.D., AND ROLLIN D. HOTCHKISS, PH.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

When rabbits are immunized with artificial antigens containing the azobenzyl glycosides of glucose and glucuronic acid, the antibodies elicited are specific and show no serological crossing (1). Since the configuration of the hexoside radicals in these antigens is identical, it is noteworthy that they give rise in each instance to immune bodies which exhibit no serological cross reactions. Differences in immunological properties must be attributed therefore to differences in the grouping occupying the sixth position in each carbohydrate radical, which in the case of the glucoside is an hydroxyl group (CH_2OH) and in the glucuronide a carboxyl group ($COOH$).

The uronic acid nucleus of the capsular polysaccharides of certain types of pneumococci appears to be of special importance in determining the serological reactions of the latter substances. In certain instances the uronic acid is glucuronic acid, while in others galacturonic acid is found as an integral part of the polysaccharide molecule. It seemed advisable, therefore, to compare the serological properties of artificially compounded antigens containing glucuronic and galacturonic acids, for in this manner it should be possible to ascertain the relationship between stereoisomerism and the immunological properties of the polar carboxyl group.

Chemical Methods

Tetracetyl p-Nitrobenzyl β-Galactoside.—This glycoside was prepared by shaking 7.7 gm. of acetobromogalactose (2) with 5.8 gm. of silver oxide and 5.8 gm. of p-nitrobenzyl alcohol in 80 cc. of ether for 1 hour. After filtering and concentrating the solution *in vacuo*, the oily residue was taken up in hot 50 per cent alcohol.

The glycoside crystallized as the solution was slowly cooled.   3.0 gm. of glycoside were recovered.   The substance was recrystallized several times from 50 per cent alcohol.   The pure substance melted at 99.5–100.5° (uncorrected).

Rotation.—$[\alpha]_D^{22°} = -35.9°$ in $CHCl_3$ (C = 1 per cent).

Analysis.—$C_{21}H_{25}O_{12}N$.   Calculated.   C 52.2, H 5.2, $COCH_3$ 35.6.
                              Found.   C 52.6, H 5.4, $COCH_3$ 35.6.

p-Nitrobenzyl β-Galactoside.—17.1 gm. of tetracetyl *p*-nitrobenzyl galactoside were suspended in 100 cc. of methyl alcohol and deacetylated with 1/30 mole of barium methylate according to the method of Isbell (3).   After removing the barium by adding the equivalent quantity of N/1 sulfuric acid, the glycoside was recovered from the mother liquors.   9.9 gm. of glycoside were obtained.   The product, recrystallized from methyl alcohol, melted at 161–162° (uncorrected).

Rotation.—$[\alpha]_D^{24°} = -32.9°$ in CH_3OH (C = 1.5 per cent).

Analysis.—$C_{13}H_{17}O_8N$.   Calculated.   C 49.5, H 5.4, N 4.4.
                              Found.   C 50.2, H 5.6, N 4.3.

p-Aminobenzyl β-Galactoside.—2.0 gm. of *p*-nitrobenzyl galactoside were dissolved in 100 cc. of absolute methyl alcohol and reduced catalytically (4).   On concentrating the alcoholic solution and dissolving the residual oil in 95 per cent ethyl alcohol, the glycoside crystallized as needles melting at 89–90° (uncorrected).

Rotation.—$[\alpha]_D^{23°} = -50.5°$ in CH_3OH (C = 0.8 per cent).

Analysis.—$C_{13}H_{19}O_6N$.   Calculated.   C 54.7, H 6.7, N 4.9.
                              Found.   C 54.6, H 6.7, N 4.6.

p-Nitrobenzyl β-Glycoside of Triacetyl Galacturonic Acid Methyl Ester.[1]—18.0 gm. of triacetylbromo galacturonic acid methyl ester (5) were dissolved in 450 cc. of anhydrous ether.   8.4 gm. of *p*-nitrobenzyl alcohol and 6.4 gm. of silver oxide were added.   The mixture was shaken for 3 hours at 24°C. until the ethereal solution no longer gave a test for the bromo compound.   The glycoside crystallized as the reaction progressed.   The mixture was filtered, the filtrate discarded, and the glycoside extracted from the residue of silver salts with chloroform.   After evaporating the latter, the glycoside crystallized on dissolving the residue in methyl alcohol.   8.2 gm. of the product were obtained as glistening needles melting at 120–122° (uncorrected).

Rotation.—$[\alpha]_D^{25°} = -27.8°$ in $CHCl_3$ (C = 1.5 per cent).

Analysis.—$C_{13}H_{11}O_8N(COCH_3)_3(OCH_3)$.

                    Calculated.   C 51.2, H 5.0, OCH_3 6.6.
                    Found.   C 51.2, H 5.2, OCH_3 6.6.

p-Nitrobenzyl β-Glycoside of Galacturonic Acid Methyl Ester.—6.0 gm. of the acetylated glycoside were suspended in 75 cc. of methyl alcohol freshly distilled over sodium.   1.5 cc. of N/1 barium methylate were added at 0° and the mixture

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[1] The galacturonic acid used in this research was furnished us by the Department of Agricultural Chemistry of the University of Wisconsin through the courtesy and generous cooperation of Dr. Karl P. Link.

sbaken at 0° for 1 hour. A second portion of 1.5 cc. of barium methylate was added and the shaking continued until all of the glycoside had dissolved. The solution stood at 0° overnight. A small amount of yellowish precipitate settled on standing and was removed. The solution was now concentrated *in vacuo* and the residue taken up in hot alcohol, and the latter allowed to cool. 2.8 gm. of the *p*-nitrobenzyl β-glycoside of galacturonic acid methyl ester were recovered. The compound crystallizes as glistening needles melting sharply at 166.5–167.5° (uncorrected).

Rotation.—$[\alpha]_D^{25} = -53.4°$ in CH_3OH (C = 0.6 per cent).

Analysis.—$C_{13}H_{14}O_8N(OCH_3)$. Calculated. OCH_3 9.0, N 2.45.
                                          Found. OCH_3 8.8, N 2.60.

p-Aminobenzyl β-Glycoside of Galacturonic Acid Methyl Ester.—0.9 gm. of the nitrobenzyl glycoside was dissolved in 50 cc. of methyl alcohol and reduced catalytically with 25 mg. platinum oxide and hydrogen. The reduction was complete in 10 minutes and the theoretical quantity of hydrogen had been utilized. The platinum was filtered off and the filtrate concentrated *in vacuo* and dissolved in a small amount of ethyl alcohol. The glycoside crystallized as well defined long silky white needles. 0.65 gm. was recovered. The compound apparently crystallizes with a half molecule of ethyl alcohol of crystallization. The melting point of the derivative is not sharp. The compound collapses at 76° and melts at 90°. A methoxyl determination shows that the derivative contains ½ mole of alcohol of crystallization. When the derivative is crystallized from a small amount of water, and the substance dried *in vacuo* over calcium chloride at room temperature, the glycoside is obtained as white well defined needles which melt at 108–110° with effervescence.

Rotation.—$[\alpha]_D^{25} = -75.8°$ in H_2O (C = 1 per cent).

Analysis.—$C_{13}H_{16}O_6(OCH_3)$. Calculated. OCH_3 9.9, N 4.5.
                                          Found. OCH_3 9.6, N 4.2.

p-Aminobenzyl β-Glucoside and β-Glucuronide.—These derivatives were prepared by methods described in the previous study (1).

Immunological Reactions

Methods.—Immunizing antigens were prepared by combining the diazonium derivatives of *p*-aminobenzyl β-glycosides of glucose, galactose, glucuronic, and galacturonic acids with normal horse serum globulin. The galacturonic acid antigen was prepared from the *p*-aminobenzyl galacturonide methyl ester by first hydrolyzing the ester with one equivalent of normal sodium hydroxide. The resulting sodium salt of the acid was diazotized and coupled to serum globulin in the usual way.

The method of immunization of rabbits and the technique of the precipitin and inhibition tests were the same as described in previous studies. Test antigens were prepared by combining the glycosides to the protein of chicken serum in order to avoid protein cross reactions. For the sake of brevity, immunizing antigens

will be referred to in the tables as glucose-globulin, glucuronic acid-globulin etc., whereas the test antigens are referred to as glucose-chick, glucuronic acid-chick, etc.

Specific Precipitin and Inhibition Tests.—The sera of rabbits immunized with azoprotein antigens prepared from the diazonium derivatives of the *p*-aminobenzyl glycosides of glucose, galactose, glucuronic and galacturonic acids were first tested for the presence of homologous

TABLE I

Precipitins in Sera of Rabbits Immunized with Glucose, Galactose, Glucuronic Acid, and Galacturonic Acid Antigens

Antiserum prepared by immunization with	Test antigen used	Final dilution of test antigen		
		1:10,000	1:20,000	1:40,000
Glucose-globulin	Glucose- chick	+++	+++	++±
	Galactose "	±	0	0
	Glucuronic acid-chick	(0	0	0
	Galacturonic " "	0	0	0
Galactose-globulin	Glucose- chick	±	0	0
	Galactose "	+++±.	+++	++±
	Glucuronic acid-chick	0	0	0
	Galacturonic " "	0	0	0
Glucuronic acid-globulin	Glucose- chick	±	±	0
	Galactose "	0	0	0
	Glucuronic acid-chick	+++	+++	++
	Galacturonic " "	±	±	0
Galacturonic acid-globulin	Glucose- chick	±.	0	0
	Galactose "	0	0	0
	Glucuronic acid-chick	±	±	0
	Galacturonic " "	++±	+++	+++

and heterologous precipitins. The results of these tests are summarized in Table I, in which it is seen that each antigen gives rise to antibodies which are distinct and specific. The antisera in each instance show little or no serological crossing. The specificity of these serological reactions is further emphasized by the specific inhibition tests, the results of which are given in Table II. It is evident that the precipitin reactions are inhibited only by the homologous, and not by any of the heterologous glycosides.

In the first paper of this series it was shown that the specificity of conjugated carbohydrate-protein antigens containing glucose and galactose is determined by differences in the spatial configuration of a single asymmetric carbon atom in the carbohydrate radical (6).   The

TABLE II

Inhibition of Precipitin Reactions of Glucuronic Acid, Galacturonic Acid, Glucose, and Galactose Antigens in Homologous Antisera

Antiserum prepared by immunization with	0.9% NaCl	u/15 p-aminobenzyl glycoside of				Test antigen used (1:5000)	Result
		Glucuronic acid*	Galacturonic acid*	Glucose	Galactose		
0.2 cc.	cc.	cc.	cc.	cc.	cc.	0.5 cc.	
Glucuronic acid-globulin	0.3					Glucuronic acid-chick	+++
		0.3					0
			0.3				+++
				0.3			+++
					0.3		+++
Galacturonic acid-globulin	0.3					Galacturonic acid-chick	+++
		0.3					+++
			0.3				0
				0.3			+++
					0.3		+++
Glucose-globulin	0.3					Glucose-chick	+++
		0.3					+++±
			0.3				+++±
				0.3			0
					0.3		+++
Galactose-globulin	0.3					Galactose-chick	++++
		0.3					++++
			0.3				+++++
				0.3			++++
					0.3		0

* Used as sodium salt.

stereochemical pattern of the asymmetric carbon atoms of glucose and glucuronic acid is identical, yet antigens prepared from the amino-benzyl glycosides of these two hexoses give rise in rabbits to antibodies which show no serological crossing.   Differences in the immunological properties of these two antigens must be attributed therefore to differ-

ences in polarity of the grouping occupying the 6th position in each carbohydrate radical. Thus a new and important factor should be taken into consideration in understanding the specificity of carbohydrates, namely the polarity of groupings within the molecule. The results given in Tables I and II show that the glucuronic and galacturonic acid antigens do not cross react appreciably and that in no instance do heterologous glycosides inhibit the homologous reactions. It may be concluded that antigens containing stereoisomeric uronic

TABLE III

Precipilin Reactions of Glucuronic and Galacturonic Acid Antigens in Antipneumococcus Horse Sera Types I, II, III, and VIII

Antipneumococcus horse serum Type	Test antigen used	Final dilution of test antigen				
		1:10,000	1:50,000	1:250,000	1:500,000	1:1,000,000
I	Galacturonic acid-chick	+++	++±	+±	+	±
	Glucuronic " "	±	±	0	0	0
II	Galacturonic " "	±	0	0	0	0
	Glucuronic " "	+++	+++	+±	±	0
III	Galacturonic " "	++±	+±	+	±	0
	Glucuronic " "	++++	+++	++	+	±
VIII	Galacturonic " "	++	+	0	0	0
	Glucuronic " "	++±	++±	++	±	±
Normal horse serum	Galacturonic " "	0				
	Glucuronic " "	0				

acids display a specificity as sharply defined as that exhibited by simple hexoside antigens. It should be emphasized, furthermore, that the immune response of rabbits is directed toward the carbohydrate radical as a whole, and not toward any individual grouping in particular.

Precipilin Reactions of Glucuronic and Galacturonic Acid Antigens in Antipneumococcus Horse Sera Types I, II, III, and VIII.—In previous work it has been demonstrated that the capsular polysaccharides of Types III and VIII pneumococci are constituted from molecules of

glucose and glucuronic acid (7). Azoprotcin antigens containing glucuronic acid react in high dilutions with antipneumococcus horse scra Types II, III, and VIII, whereas the corresponding glucose antigen shows little or no serological activity (1). Since it is now known that the capsular polysaccharide of Type I Pneumococcus contains galacturonic acid (8), one might expect the artificial galacturonic acid antigen to precipitate in antipneumococcus horse serum Type I. That this is the case is seen from the results given in Table III. The galacturonic acid antigen precipitates, however, in Types III and VIII serum as well. This reaction might be explained by assuming that Types III and VIII pneumococcus contain a galacturonic acid constituent which gives rise to antibodies reactive with the

TABLE IV

Precipitin Reactions of Glucuronic and Galacturonic Acid Antigens in Antipneumococcus Horse Serum Type III before and after Absorption with the Homologous Capsular Polysaccharide

Antipneumococcus horse serum Type III	Test antigen used	Final dilution of test antigen		
		1:5000	1:20,000	1:80,000
Unabsorbed	Glucuronic acid-chick	++++	+++	++±
	Galacturonic " "	++	++	+±
Absorbed with SSS III	Glucuronic " "	0	0	0
	Galacturonic " "	0	0	0

artificial antigen. When antiserum is first absorbed with homologous capsular polysaccharide, however, the absorbed serum fails to react with the galacturonic acid antigen, as shown in Table IV. It is obvious, therefore, that the reaction of the galacturonic acid antigen in antipneumococcus horse serum Type III (and probably in Type VIII as well) is one which takes place between the antigen and the type specific carbohydrate immune body.

Since the results of the present study demonstrate that antigens containing isomeric uronic acids give rise in rabbits to specific antibodies, it is difficult to understand the reaction of galacturonic acid antigen with immune bodies elicited by immunization of horses with organisms containing glucuronic acid in the encapsulating poly-

saccharide. It is believed, however, that the precipitation of galacturonic acid antigen in antipneumococcus horse sera Types III and VIII is of a non-specific nature. Evidence for this hypothesis is provided by the results of the specific inhibition tests given in Table V. The reaction of glucuronic acid antigen in Types II, III, and VIII antipneumococcus horse sera is inhibited only by the glucuronide, whereas

TABLE V

Inhibition of Precipitin Reactions of Glucuronic and Galacturonic Acid Antigens in Antipneumococcus Horse Sera Types I, II, III, and VIII

Antipneumo-coccus serum Type	0.9% NaCl	M/15 p-aminobenzyl glycoside of		Test antigens (1:10,000		Result
		Glucuronic acid	Galacturonic acid	Glucuronic acid-chick	Galacturonic acid-chick	
0.2 cc.	cc.	cc.	cc.	cc.	cc.	
I	0.3				0.5	+++
		0.3			0.5	+++
			0.3		0.5	0
II	0.3			0.5		+++
		0.3		0.5		0
			0.3	0.5		+++
III	0.3			0.5		+++±
		0.3		0.5		0
			0.3	0.5		+++±
	0.3				0.5	++±
		0.3			0.5	0
			0.3		0.5	0
VIII	0.3			0.5		+++
		0.3		0.5		0
			0.3	0.5		+++
	0.3				0.5	++
		0.3			0.5	0
			0.3		0.5	0

the precipitation of galacturonic acid antigen is inhibited by either uronide. On the other hand, the galacturonic acid antigen, which contains the uronic acid found in the Type I polysaccharide, precipitates in Type I antipneumococcus serum even in the presence of the glucuronide. The precipitation is of course inhibited by homologous galacturonide.

TABLE VI

Precipitin Reactions of p-Aminobenzene Sulfonic and Carboxylic Acid Antigens in Antipneumococcus Horse Sera Types I, II, III, and VIII

Antipneumococcus horse serum Type	Test antigen used	Final dilution of test antigen						
		1:1000	1:2500	1:5000	1:10,000	1:20,000	1:40,000	1:80,000
I	Carboxylic acid-chick*	+++	++	±	0	0	0	0
	Sulfonic " "	+++	++±	++	+±	0	0	0
II	Carboxylic " "	0	0	0	0	0	0	0
	Sulfonic " "	+	+±	0	0	0	0	0
III	Carboxylic " "	++++±	++++	++++±	++++±	+++±	++±	+
	Sulfonic " "	+++	++++	++++	+++±	++	±	0
VIII	Carboxylic " "	++++±	++++±	+++±	+±	±	0	0
	Sulfonic " "	+++	++++	+++	++±	++	+	±
Normal horse serum	Carboxylic " "	0	0	0	0	0	0	0
	Sulfonic " "	0	0	0	0	0	0	0

* Test antigens prepared by combining diazotized p-aminobenzene sulfonic and carboxylic acids with chicken serum.

Evidence has been presented indicating that the active acidic groups of bacterial carbohydrates and the free amino groups of antibody are involved in the specific precipitation of homologous immune protein (9). Similarly, the precipitation of glucuronic and galacturonic acid

TABLE VII

*Inhibition of Precipitin Reactions of Acid Azoprotein Antigens in Antipneumococcus Horse Serum Type III**

Test antigen used (1:5000)	0.9% NaCl	0.2 molar inhibiting substance				Result
		p-Amino-benzyl glucuronide	p-Amino-benzyl galacturonide	p-Amino-benzene sulfonic acid	p-Amino-benzene carboxylic acid	
0.5 cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	
Glucuronic acid-chick	0.3					++++
		0.3				0
			0.3			+++
				0.3		++++
					0.3	++++
Galacturonic acid-chick	0.3					+++
		0.3				0
			0.3			0
				0.3		±
					0.3	0
Sulfonic acid-chick	0.3					+++±
		0.3				0
			0.3			0
				0.3		0
					0.3	0
Carboxylic acid-chick	0.3					+++
		0.3				0
			0.3			0
				0.3		±
					0.3	0

* 0.2 cc. of antipneumococcus horse serum Type III used in each test. All inhibiting substances used as neutral sodium salts.

antigens in antipneumococcus horse sera might be attributed to a reaction between the acidic groups of the antigen and the basic groups of the antibody protein molecule. Artificial azoprotein antigens containing organic acid radicals, quite unrelated in chemical constitu-

tion to the uronic acids, might likewise be expected to precipitate in antipneumococcus horse sera.

Antigens prepared from *p*-aminobenzene carboxylic and sulfonic acids, though not reactive in normal horse serum, precipitate in antipneumococcus sera, as seen from results of precipitin tests given in Table VI. The precipitation of these antigens in antipneumococcus serum Type III is inhibited indiscriminately by the sodium salt of any one of the uncombined acidic derivatives, as the results of the specific inhibition tests in Table VII show. The reaction of glucuronic acid antigen in the immune horse serum is inhibited, however, only by the glucuronide and not by any of the heterologous acid derivatives. The precipitation of glucuronic acid antigen in Type III antipneumococcus

TABLE VIII

Precipitin Reactions of p-Aminobenzene Sulfonic and Carboxylic Acid Antigens in Antipneumococcus Horse Serum Type III before and after Absorption with the Homologous Capsular Polysaccharide

Antipneumococcus horse serum Type III	Test antigen used	Final dilution of test antigen	
		1:5000	1:10,000
Unabsorbed	*p*-Aminocarboxylic acid-chick	+++±	+++
	p-Aminosulfonic " "	+++±	+++
Absorbed with SSS III	*p*-Aminocarboxylic " "	0	0
	p-Aminosulfonic " "	0	0

horse serum may be regarded therefore as approaching more closely the homologous reaction. The reaction of all these acid antigens in antipneumococcus horse serum Type III (and probably in antisera of other types as well) represents a precipitation of antigen and immune protein, for precipitation does not occur in normal horse serum, nor in immune serum from which the type specific antibody has been removed by absorption. The results of these tests are given in Table VIII.

The azoprotein antigens containing the galacturonide, or the aromatic acid radicals, possess but one property in common, namely acidic groups of divergent nature. It does not appear illogical to assume, therefore, that the reaction of these antigens in antipneumococcus horse sera represents a neutralization of the charge of basic groups

of the antibody protein by the acid groups of the hapten, followed by precipitation.

In conclusion it should be pointed out that none of the acid azo-protein antigens precipitate in antipneumococcus rabbit sera. The reason for this is not understood. It is apparent, however, that certain fundamental differences exist between the antibodies of the horse and the rabbit (10). The great affinity of acid antigens for the antibodies of immune horse sera and their failure to precipitate in the corresponding rabbit antisera represent another striking difference between the immune protein of these two animal species.

DISCUSSION

In view of our present knowledge concerning the constitution of hexoses (11) and hexose uronic acids (12), it may be stated with fair certainty that the p-aminobenzyl β-glycosides of glucose, galactose, glucuronic acid, and galacturonic acid are pyranoside derivatives. The configuration of these substances can therefore be represented by the following formulae in which R signifies the p-aminobenzyl group— $CH_2C_6H_4NH_2$.
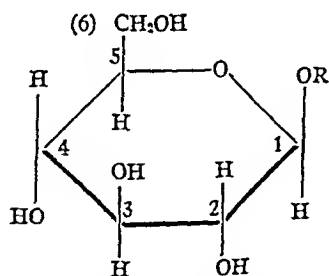
p-Aminobenzyl β-glucoside
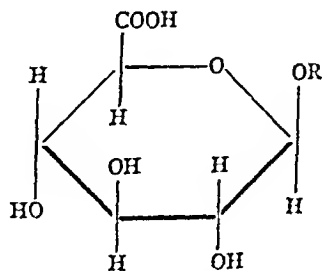
p-Aminobenzyl β-glucuronide
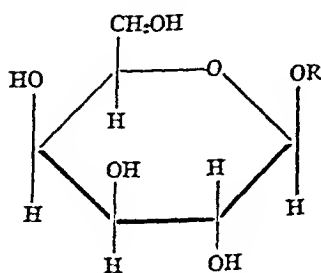
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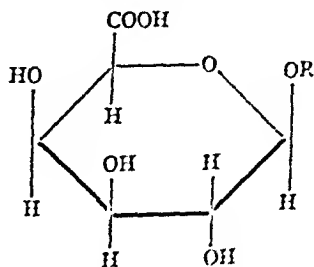
p-Aminobenzyl β-galacturonide

As indicated earlier in this communication, both the glucoside and glucuronide have an identical configuration and differ only in the grouping occupying the 6th position; this relationship is true also of the galactoside and galacturonide. The glucoside and glucuronide differ structurally from the galactoside and galacturonide, however, in the spatial configuration of the H and OH groups occupying the 4th carbon atom. In the first communication of this series (6) it was shown that in antigens containing simple hexosides a rotation of the H and OH groups through an angle of 180° on carbon atom 4 of the glycoside radical sufficed to confer a distinct and individual specificity upon each. That this is true of the corresponding uronides is now evident from the results of the foregoing serological analysis. This fact is important, for it undoubtedly finds application in understanding the specificity of the naturally occurring bacterial polysaccharides, which in certain instances contain glucuronic acid, and in others galacturonic acid. In a previous study on the specificity of artificial antigens containing glucose and glucuronic acid (1) it was found that a change in polarity of the terminal grouping of a simple carbohydrate suffices to confer a distinct specificity upon each. This fact is further substantiated by the results of the present communication in which it is seen that antigens containing galactose and galacturonic acid give rise likewise to antibodies which are specific and show no serological crossing.

That antigens containing glucuronic and galacturonic acids, each containing a polar carboxyl group, give rise in rabbits to specific antibodies, emphasizes anew the fact that the spatial relationship of the lesser polar hydroxyl (OH) groups suffices to determine the serological specificity of carbohydrates. It should be emphasized, however, that in the rabbit the specificity of the antibody to which these artificial carbohydrate-protein antigens give rise is determined in all instances by the glycoside radical as a whole and not by any particular grouping within the carbohydrate molecule. It appears at first difficult to understand the precipitation of simple uronic acid antigens in antipneumococcus horse sera and their failure to precipitate in the corresponding immune rabbit sera. It must be remembered, however, that in these instances we are dealing with the sera of different animal species, the

immune protein of which may differ considerably in chemical properties. Furthermore we know as yet but little of the structure of the pneumococcus polysaccharides in which certain of the hydroxyl groups are known to be in chemical combination, whereas others may be masked by steric hindrance effects not obtaining in the simple monosaccharides. It appears, however, that the antibodies to which the polysaccharides give rise, in horses at least, are directed toward the molecule as a whole, and toward the uronic acid nucleus as well.

Antigens containing benzene carboxylic and sulfonic acid radicals, quite unrelated in chemical constitution to the pneumococcus polysaccharides, precipitate vigorously in antipneumococcus horse sera Types I, III, and VIII. These reactions are in each instance inhibited non-specifically by the glycosides of uronic acids and by the sodium salts of the uncombined benzene sulfonic and carboxylic acids themselves. It is evident therefore that a portion of the anticarbohydrate globulin of pneumococcus horse serum is capable of reacting with antigens containing acid groups of widely divergent nature. The chemical properties of the immune globulin must differ from those of the globulin of normal horse serum, for precipitation of these acid antigens does not occur in normal serum. The reactive groups of the immune protein with which these azoproteins combine, are believed to be basic groups which may unite not only with the acidic groups of the homologous polysaccharide, but which can combine non-specifically with the acidic groups of the azoproteins in question. This concept is supported by the results of experiments now being carried out, in which it has been found that partial acetylation of the amino groups of Type III pneumococcus antibody deprives the latter of the ability to precipitate these same acid-containing azoproteins.

In conclusion, it should be said that the highly selective immunological specificity of bacterial polysaccharides can be understood only when a more complete picture of their chemical constitution has been gained.

SUMMARY

1. Azoprotein antigens containing glucuronic and galacturonic acids give rise in rabbits to specific antibodies. The immune sera show no serological crossing with antigens containing glucose or galactose.

2. The galacturonic acid antigen reacts in antipneumococcus horse serum Type I in high dilutions.

3. Azoprotein antigens containing galacturonic acid, benzene sulfonic and carboxylic acids precipitate in antipneumococcus horse sera of various types but not in normal horse serum.   The mechanism underlying these cross reactions is discussed.

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STUDIES ON THE PHYSIOLOGICAL CONDITIONS
PREVAILING IN TISSUE CULTURES

By HANS ZINSSER, M.D., AND EMANUEL B. SCHOENBACH,[*] M.D.

(From the Department of Bacteriology and Immunology, The Harvard Medical School, Boston)

The method for cultivating virus agents which was published by Maitland and Maitland (1) in 1928 may be regarded as having provided one of the most important techniques now available for the study of ultramicroscopic virus agents outside the animal body. In the original method, vaccinia virus was induced to multiply in a medium consisting of minced hens' kidney, serum and Tyrode solution. The work of the Maitlands was soon confirmed by Rivers, Haagen and Muckenfuss (2) and by Eagles and McClean (3). Although no fundamental changes have been made in the original technique, minor modifications, such as the use of Tyrode solution and tissue alone, the addition of animal sera of various kinds and of tissues of different origins, have been practiced by many observers. The method has also become useful in the cultivation of Rickettsiae by the modification first suggested by Nigg and Landsteiner (4), who used tunica vaginalis tissue of guinea pigs and guinea pig serum, with or without Tyrode solution.

In both cases, success or failure with the Maitland method has, so far, depended largely upon empirical adjustments and modifications of minor factors. Both in virus and in Rickettsia cultivation it has been found that it was essential to avoid the use of excessive amounts of tissue. But although many virus agents can be successfully grown in flasks closed with the ordinary cotton plugs, Rickettsia multiplication seems to take place best when the flasks are tightly stoppered, provided a suitable adjustment is made between culture volume and air space. Moreover, a fundamental physiological difference between the

growth requirements of the two classes of infectious agents is indicated by the fact that, whereas virus growth reaches its maximum usually between the 3rd and 4th days, at a time when, according to Rivers, Haagen and Muckenfuss, the tissue is still alive, Rickettsia multiplication appears to speed up after the 6th, 7th or 8th day when presumably tissue viability and, consequently, metabolism have come to a standstill.   The supposed limitation of Rickettsia growth to specific mammalian tissue is no longer tenable, since Miss FitzPatrick, in our laboratory, has recently obtained growth of the murine organisms in Maitland cultures containing embryonic chick tissue with chick serum-Tyrode solution.   These experiments will be separately reported.

No one has so far succeeded in growing any of the virus agents or Rickettsiae in cultures other than those prepared by one or the other of the "tissue culture" methods.   While we do not underestimate the importance of the interesting observations of Eagles (5) with tissue extracts, it does not seem to us that his experiments can as yet be regarded as a successful elimination of the tissue factor from virus cultures.   The presence of cells is, up to the present time, essential to any considerable degree of virus or Rickettsia multiplication.   And since accumulated evidence is strongly in support of the assumption that, within the infected body, both these infectious agents multiply intracellularly, it is likely that the cells, either directly or indirectly, determine the conditions which permit growth.

The experiments here recorded represent an attempt to determine some of the measurable physiological changes which take place in Maitland cultures.

Our work has included the study and correlation of: (a) changes of reaction; (b) tissue viability; (c) potential developed at platinum electrodes, and (4) oxygen utilization.

We are indebted to Dr. Herald Cox for our original strain of the equine encephalitis virus which was used throughout.

Changes of Reaction

The recorded pH values were all determined colorimetrically by use of the Clark standards.   Within the ranges of pH 7 to pH 8.2 a scale of standard colors with phenol red, made up according to the method of Medalia (6), was used. Below and above this range the reactions were determined on porcelain spot

plates by the use of several overlapping indicators. The measurements may be considered accurate within 0.2 pH unit.

The reaction of the Maitland tissue cultures made with a Tyrode solution[1] containing 0.5 gm. of sodium bicarbonate per liter is usually about pH 7.4 immediately after filtering and mixing. When serum, either chick serum or horse serum, is added to these cultures in the proportion of one part of serum to three of Tyrode, the reaction is little changed, but occasionally is slightly less alkaline, pH 7.0 to 7.2.

When the ordinary small amounts of either chick embryo or minced guinea pig tunica are employed, that is, amounts not exceeding 1 drop of a moist tissue suspension[2] to not more than 3.5 cc. of fluid medium, in open flasks, the initial pH of 7.4 to 7.6 increases within 3 days to from 7.8 to 8.4 and higher. In similar flasks, closed, the initial pH usually dropped to below 7.0, and on occasion was found as low as 6.2 to 6.5.

When two or three times the ordinary amounts of tissue are used, both open and closed flasks turn acid, ending with final reactions at or about pH 6.0.

Changes in reaction are thus functions of the amounts of tissue added and of the freedom with which the produced carbon dioxide can escape; that is, whether the flasks are stoppered with cotton or are tightly closed. In flasks tightly stoppered and observed over the course of at least 2 weeks, as in Rickettsia cultivation, the initial pH of 7.4 to 7.6, after a temporary slight rise of alkalinity on the 3rd or 4th day, usually falls to about pH 7.0, rarely below this. It is quite probable that this relatively slight change, in spite of the closure of the flasks, can be accounted for by the fact that the amounts of tissue used in Rickettsia cultures are invariably small and that guinea pig tunica tissue is metabolically less active than embryonic chick tissue.

The growth of equine encephalitis virus in chick embryo Maitland cultures usually leads to a relatively high alkalinity within 3 days, owing, we believe, to the fact that the tissue is partly killed and its CO_2 production thereby interrupted.

[1] The Tyrode solutions used contained NaCl 8.0, KCl 0.2, CaCl$_2$ 0.2, MgCl$_2$ 0.1, NaH$_2$PO$_4$ 0.05, NaHCO$_3$ 0.5 or 1.0 for typhus, glucose 1 gm., 1000 H$_2$O.

[2] This amount of tissue corresponds approximately to 0.16 to 0.18 mg. of total nitrogen.

TABLE I

*Viability of Chick Tissue in Maitland Cultures**

The following tabulation is compiled from a great many experiments carried out in the course of our work. We have not tried to complete it systematically, since the general averages here given gave us the information we desired to correlate viability with other factors.

Description	Opened or closed	Period of viability of tissue	Average final reactions
In Tyrode solution alone			
Culture optimum amounts of tissue	Cotton stoppers	Usually 6 days	pH 7.8–8.0
Culture optimum amounts of tissue	Rubber "	Rarely 6 days	pH 6.8–7.2
Larger amounts of tissue	Cotton "	3 to 5 days, depending on reaction	Approaching pH 6.0
Chick serum-Tyrode 1 to 3			
Culture optimum amounts of tissue	" "	6 days	pH 7.8
Culture optimum amounts of tissue	Rubber "	4 to 5 days	pH 7.0–7.2
Larger amounts of tissue (3 times above)	Cotton "	Dead on 3rd day	pH 5.8–6.0
Culture amounts with virus	" "	2 to 3 days	pH 7.8–8.0
Horse serum-Tyrode 1 to 3			
Culture amounts chick tissue	" "	Up to 4 days	pH 7.0
Culture amounts chick tissue with virus	" "	Up to 3rd day slight growth	pH 7.4
Fresh tissue, "old" Tyrode	" "	At least 3 days	pH 6.3–6.8
Old tissue, new Tyrode	" "	6 days (total)	Alkaline
Tissue heated 50°C. for 10 min.		No growth	

In almost all cases in which final pH ranged from 6 to 6.4 or below, the tissue either grew in only a few of the spots or not at all. Alkalinity, even up to 9.6, did not kill tissue.

* Viability was determined by "spotting" pieces of tissue in heparinized chicken plasma in sealed Petri plates.

In a later section, it will be seen that the viability of tissue may persist in spite of shifts to the alkaline side up to and above pH 8.0.

In two cases, tissue plants showed some growth when taken from a flask having a final reaction of pH 9.6.   Reactions approaching pH 6.0 are more unfavorable for tissue survival.   Observation of changes in reaction suggest that the empirically observed necessity of avoiding excessive amounts of tissue in the Maitland technique depends upon excessive shifts to the acid side.   It is not impossible that larger and more rapid yields of virus might be produced if cultures could either be buffered or daily readjusted with small amounts of alkali.   The favorable effect of reasonable amounts of serum may be partially a result of its buffering effect.   Experiments in these directions have not yet been completed.

Determinations of Potential Changes in Tissue Cultures

Recent years have led to considerable interest in biological electrode potentials, and a great many publications dealing with this subject in the study of bacteria have appeared during the last 5 years.   The literature has been summarized by Hewitt (7), and the subject in general has been discussed by Clark (8).   It has been suggested by Plotz (9), that potential changes in such cultures may play an important rôle in the multiplication of virus agents.   It was largely, indeed, as a result of a discussion presented at our laboratory by Dr. Plotz that our interest in this matter was aroused.   Plotz based his views to a great extent on the work of Wurmser (10) and on the possibility, presented by the studies of Wurmser and Geloso (11), that potentials might be stabilized at various levels by the use of the "glucides" produced by the anaerobic heating of alkaline sugar solutions.

It seemed desirable, therefore, to study this factor and to develop a method for the study of electrode potentials in Tyrode tissue mixtures, with and without serum, in which the conditions established resembled as much as possible those prevailing in the ordinary Maitland technique.

We are aware of the fact that the interpretation of recorded results in terms of chemical exchange or the transfer of energy must be left for specialists.   But we felt nevertheless that a beginning in such studies must be made by bacteriologists familiar with infectious agents and cultural methods, and that adaptation of the relatively simple methods of potential measurement to a sterile cultural technique represented a bacteriological problem.   We are indebted to Professor Baird Hastings for much advice and helpful criticism.

It was necessary to devise a method in which the variable factors should be reduced to a minimum and which permitted the observation of cultures under sterile conditions during periods corresponding to those necessary for virus multiplication, that is, 3 or 4 days. We decided to attempt to make direct electrode measurements instead of using the dyes available for similar purposes, chiefly because of the
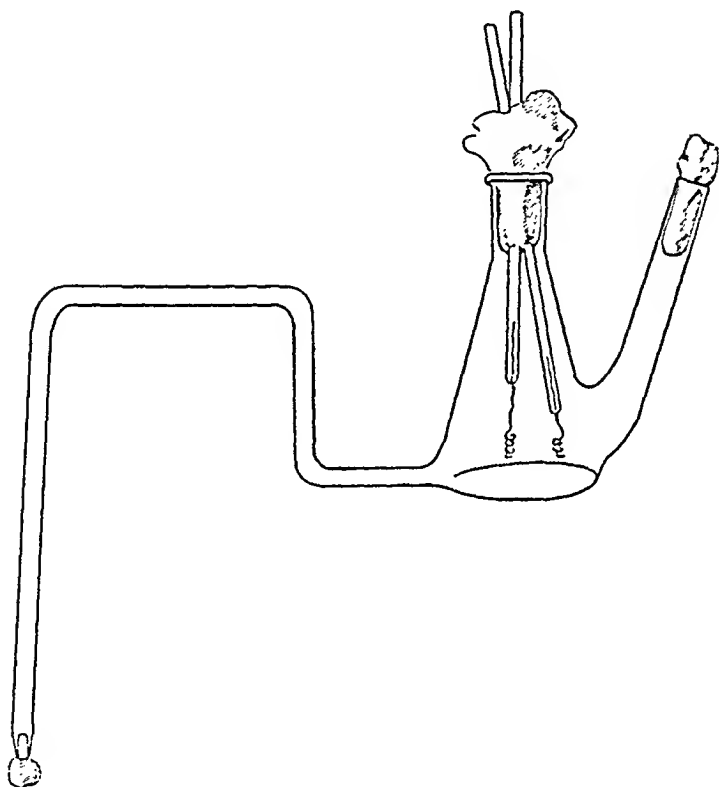
FIG. 1

possible toxicity of these substances and their consequent interference with growth.

A satisfactory technique was eventually based on the construction of the special flasks illustrated in Fig. 1. The side arms of these flasks were stoppered with cotton and two or three curled platinum wire electrodes introduced,[3] through the cotton stoppers. The flasks were then wrapped in paper and subjected to dry

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[3] Two or three electrodes were used in every flask and experiments thrown out in which serious discrepancies between individual electrodes were observed.

sterilization. Just before setting up the experiments, 4 per cent agar in distilled water, containing 2 per cent potassium chloride, was carefully introduced into the long, bent arm of each flask with a sterile syringe to which a small piece of rubber tubing with pinch cock was attached. In order to further insure sterile manipulation, the agar was put in while still very hot and run as far as the joint between the bottom of the flask and the bent arm, up to the funnel-shaped aperture connecting the two. The flask was then held steady until the agar hardened. Shrinkage during cooling was allowed for in the filling, the object being to permit free contact between the agar bridge and the culture material. The electrodes were adjusted so that they came as close as possible to the bottoms of the flasks without touching them. Hot paraffin was then run into the cotton stoppers holding the electrodes, so that these might be held firmly in place during the experiment. Finally, the tissue cultures to be studied were run into the open side arms under sterile precautions. These flasks, usually in pairs, experiment and control, were then set up in a water bath so that the bottoms of the flasks were covered with water at 37°, and the arms holding the agar bridges,—turning over the edge of the water bath,—were immersed in a saturated potassium chloride liquid junction in which was also immersed the connection of a 0.1 N calomel half-cell at the times when readings were taken. The electrodes were then connected in the usual way with a potentiometer which was the Leeds and Northrup type 7665A and a galvanometer which was the Leeds and Northrup type 2420C. The potentiometer was calibrated to 0.5 millivolts and the galvanometer had a current sensitiveness per unit scale of 0.025 micro amperes which registered 0.25 millivolts with 10,000 ohms resistance. The standard cell and the 0.1 N calomel cell were standardized for us through the courtesy of Dr. E. J. Cohn. The value of the calomel cell has been taken as 0.338 v. This value when added to the determined potential E_1 gives the potential referred to the normal hydrogen electrode and designated E_h.

The adaptation of an apparatus of this kind to tissue culture study of course necessitated the introduction of a number of factors which might easily lead to inaccuracy. Moreover, it was necessary for obvious reasons to use an isotonic KCl-agar bridge and to immerse this in a saturated KCl liquid junction. Another possible source of error was thereby introduced. In order to guard against gross inaccuracies the apparatus was checked by a quinhydrone half-cell at a known hydrogen ion concentration, placed in one of the described flasks and set up under the standard conditions of our experiments. This control showed a variation from the theoretical so slight that we felt confident that it was negligible in comparison with the range of variations obtained in actual experiment.[4]

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[4] Dr. Hastings, who has been kind enough to go over this paper for us, advises emphasis upon the point that these potential measurements are not to be confused with those found in reversible oxidation-reduction systems under anaerobic conditions. They differ essentially in that they were made under a tension of oxygen approaching that of air in a fluid medium containing various enzymes and substrates. Absolute values are not capable of being given physicochemical interpretation, certainly not by the writers.
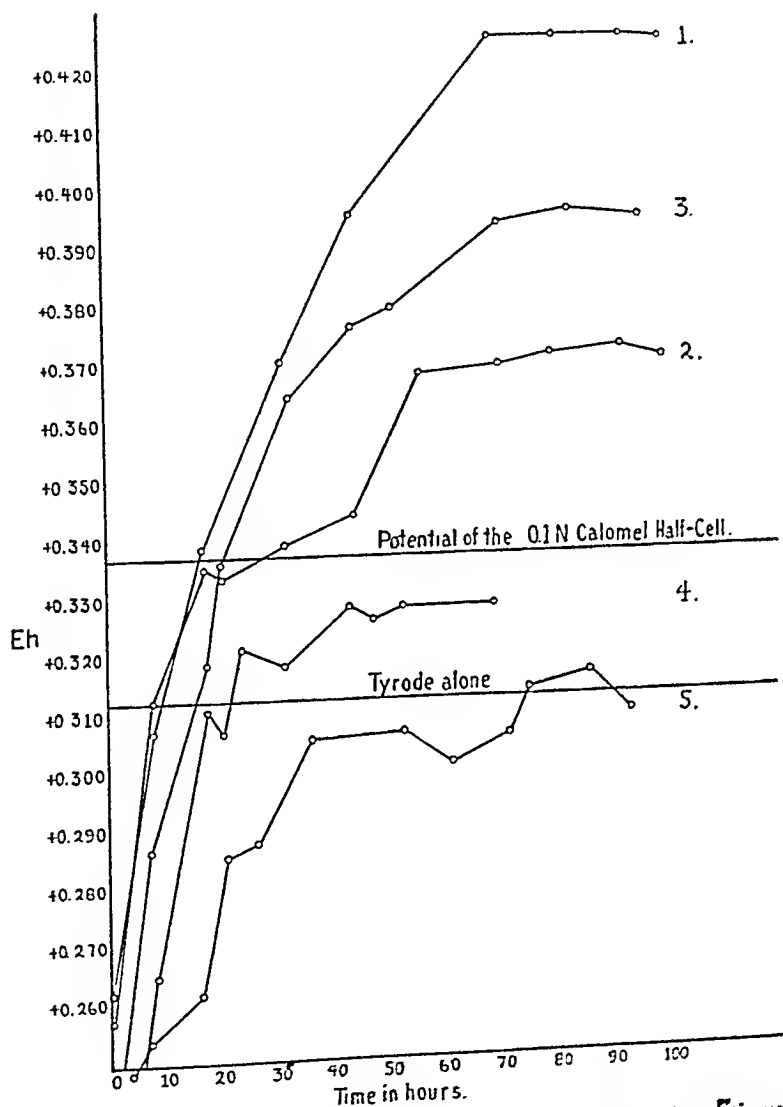
FIG. 2. Potential curve of chick tissue. Curve 1, chick embryo tissue in Tyrode alone. Curve 2, chick embryo tissue, heated to 50°C. for 10 minutes, in Tyrode alone. Curve 3, chick embryo tissue in chick serum-Tyrode. Curve 4, chick embryo tissue inoculated with equine encephalitis virus in chick serum-Tyrode. Curve 5, chick embryo tissue in horse serum-Tyrode.

In the accompanying charts, we indicate some of the results of our potential measurements. We may summarize the results of these experiments in the following way.

Potential Measurements in Cultures Containing Embryo Chick Tissue.—In mixtures of embryonic chick tissue and Tyrode solution, there is invariably a rise of potential in the order of from 80 to 110 millivolts, which flattens out in from 40 to 60 hours. In these mixtures the initial potential (E_1) is usually negative at the beginning, within the range of −0.100 to −0.040 millivolts. These figures, bowever, were subject to considerable variation, probably owing to differences in amounts of tissue and intervals between making of cultures and first readings. It is nevertheless certain that initial potentials were always relatively low and that the trend was always upward toward the positive.

Such rises of potential were observed both in cases in wbich the actual reaction rose from pH 7.4 to pH 8.6 and in those in which there was a fall of pH from 7.4 to approximately 6.4. For these reasons, it may be inferred that the rise of potentials was not directly due to reaction changes, though the actual potential levels were of course affected.

Tissue heated to 50° for 10 minutes, though no longer supporting virus growth, showing little oxygen utilization and no longer viable in plasma cultures, always exhibited a definite rise of potential similar to, though usually somewhat lower than that apparent in the cultures with unheated tissue.

In a considerable number of experiments in which chick tissue-Tyrode cultures inoculated with equine encephalitis virus were compared with uninoculated flasks, the virus appeared to lower the level of the final potential, but not to suppress the rise entirely. An example of such an experiment is the following:

Experiment.—Two "potential" flasks of chick embryo tissue in appropriate quantities in 6.0 cc. chick serum-Tyrode.

Flask 1.—Inoculated with virus. Rise of potential 122 millivolts in 36 hours. Final pH 6.9.

Flask 2.—No virus. Rise of potential, 189 millivolts. Final pH 7.2.

The inoculation of the virus flask was made from a titrated culture in such a way that the initial concentration of virus was less than 10^{-2}. The final titration was 10^{-4}, indicating not only survival but increase of the virus.

Since it was found that the equine encephalitis virus would grow almost as well in chick tissue-horse serum-Tyrode mixtures as when chick serum was used, comparative potential measurements were made in which chick tissue-horse serum mixtures were compared with similar flasks containing chick serum instead of the horse serum. The usual rise of potential was observed in the horse serum mixtures, though it started at a lower level and did not rise as high as in the controls.

Since our respiration measurements and viability tests seemed to indicate that the gradual diminution of tissue activity in Tyrode solution did not depend upon accumulation of inhibitory products of metabolism, we carried out a number of potential experiments in which "old" Tyrode solution which had been incubated with chick tissue for 3 days was used as a menstruum for fresh tissue in a potential experiment. As was to be expected, since our method directly measures the potential of the fluid and not that of the suspended tissue, the potential in these cases started high and continued to rise another 70 millivolts within the next 3 days. It is interesting in this connection to consider the fact that such "old" Tyrode still contained about 50% of its original sugar content, calculated without allowance for slight concentration by evaporation.

A converse experiment in which new Tyrode was added to old tissue showed that the potential starts at the usual low level and rises in the ordinary way.

We may conclude from these experiments that, unlike cultures of many bacteria, mixtures of physiological fluids and living cells as used for virus and Rickettsia cultivation regularly exhibit considerable rises of electromotive potential within the first 3 days of observation, and that these changes are not purely functions of changes of hydrogen ion concentration. As a rule, such rise of potential is complete between the 40th and the 90th hour, stabilizing at about the point at which respiratory exchange practically stops.

Since cultures heated to 50° for 10 minutes, which no longer utilize oxygen to any extent, support neither virus nor Rickettsia growth and contain no viable cells, still exhibit a definite rise of potential, it seems not unreasonable to assume that such changes of potential are not the sole determining processes in either virus or Rickettsia cultivation, though of course they may be contributory factors.

Moreover, just as in the respiration experiments, the potential measurements indicate that entirely different physiological conditions are involved, respectively, in virus cultures and in Rickettsia cultures; since virus multiplication takes place during the period of active exchange, and Rickettsia growth appears to speed up after these changes have become stabilized, and the cells are either no longer viable, or are at any rate in a state of reduced metabolic activity.

Experiments on Respiration

The method used for the determination of tissue respiration was carried out with the Warburg modification of the Barcroft-Haldane microrespirometer.

Flasks were used which had central wells to contain potassium hydroxide for the absorption of carbon dioxide. The directions given in the book on manometric methods by Dixon (12) were followed in the mercury standardization of volumes and for the calculation of respiratory quotient and oxygen utilization. Brodie's solution, made up by the original formula described by Warburg, was used. The density of this, as determined for us in the laboratory of Dr. Edwin Cohn, was 1.03094.

Since it was necessary to work in a sterile manner, the small flasks, with curled bits of Whatman starch-free No. 40 filter paper placed in the central KOH reservoir and stoppered with cotton, were subjected to autoclave sterilization and dried. The Tyrode solution or Tyrode-serum mixtures were run into the bottom of these flasks under sterile precautions with slightly bent pipettes, and the tissue was added in a similar manner. The ground glass joints of the manometer were then flamed with a Bunsen burner and greased with sterile anhydrous lanolin before the flasks were attached. In later experiments, the closure was reinforced with paraffin. With a little practice, this method resulted in sterile runs. The volumes of fluid used were 2 cc., but absolute uniformity of amounts of tissue were not attempted in the interests of sterility. The large number of comparative experiments carried out insured against errors of principle from this source. Type experiments are charted in Figs. 3 and 4.

We may summarize the results of a great many experiments as follows:

Oxygen Utilization of Chick Tissue in Tyrode Solution.—When chick tissue was added to Tyrode solution in amounts approximately equivalent to those used in tissue cultures, the oxygen uptake amounted in
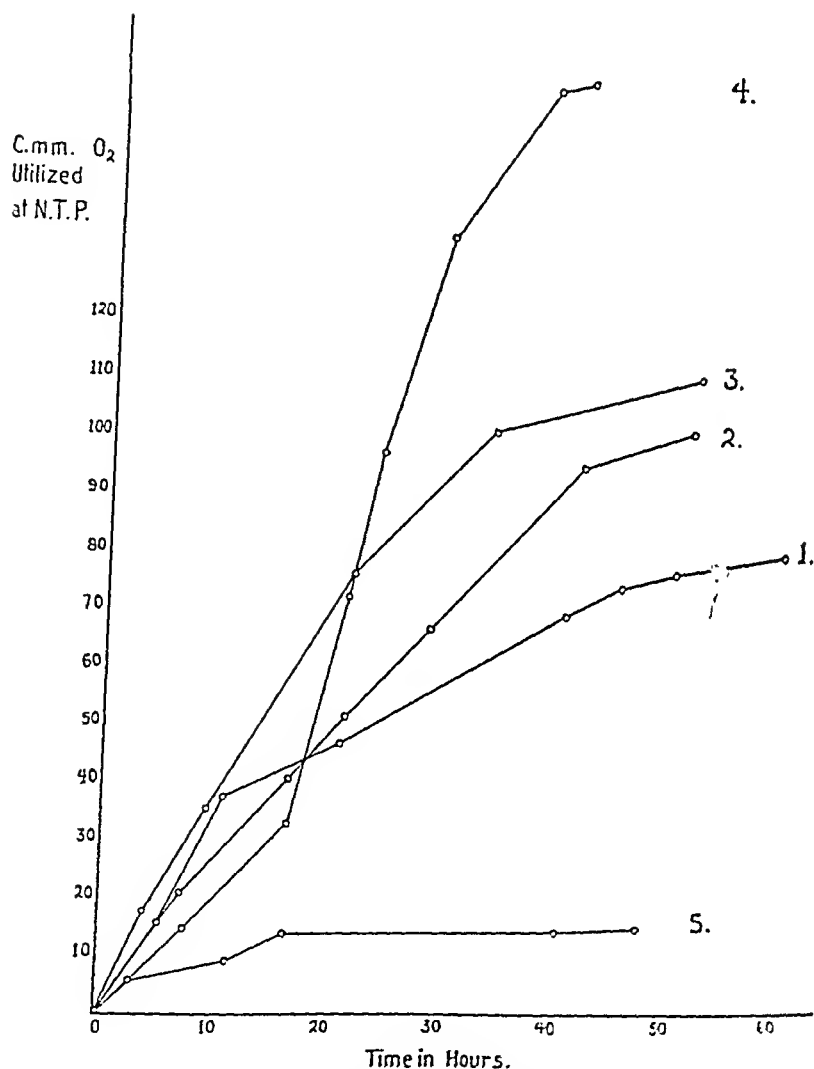
FIG. 3. Respiration of chick tissue. Curve 1, chick tissue in Tyrode alone. Curve 2, chick tissue in chick serum-Tyrode. Curve 3, chick tissue in horse serum-Tyrode. Curve 4, chick tissue in chick serum-Tyrode inoculated with equine encephalitis virus. Curve 5, chick tissue, heated to 50°C. for 10 minutes, in Tyrode alone.

rough averages to from 90 to 100 c.mm. of oxygen in from 70 to 80 hours. About 70 per cent of this uptake usually took place within the first 40 hours, at which time the oxygen uptake rapidly began to

decline, approaching a constant level of not more than about 3 c. mm. per 24 hours thereafter.

Since, of course, in the Warburg apparatus, the carbon dioxide is removed by the KOH and the cultures rapidly become alkaline, it was necessary to determine whether the oxygen uptake under these circumstances was similar to or different from that taking place in cultures kept in an incubator. To ascertain this, a series of cultures was incubated as usual and put into the Warburg apparatus after 40, 60
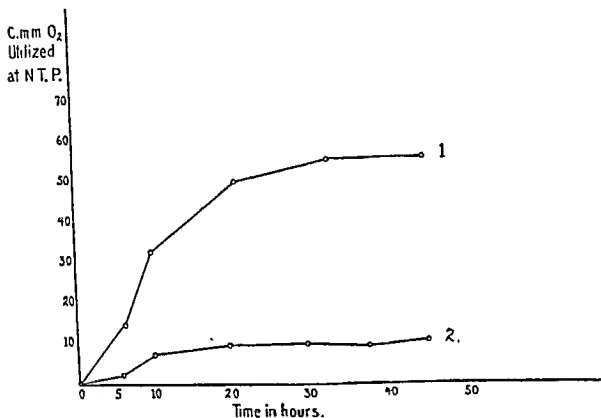
FIG. 4. Respiration of guinea pig tunica. Curve 1, guinea pig tunica in guinea pig serum-Tyrode. Curve 2, guinea pig tunica in guinea pig serum-Tyrode after heating to 50°C. for 10 minutes.

and 90 hours, respectively. The oxygen utilization in such flasks was then compared with corresponding rates observed at analogous times in Warburg flasks put into the respirometer from the beginning. It was found that the results of such "staggered" determinations corresponded approximately to those of the continuous experiments at the analogous times. Our measurements could thus be taken to represent the approximate oxygen utilization occurring in tissue culture flasks incubated in the ordinary way.

After respiration had ceased, a change of Tyrode solution, while

slightly stimulating renewed respiration, did not lead to complete resuscitation. Spot cultures in plasma showed the tissue to be still viable after 96 hours, the point at which the Tyrode was renewed.

Comparative respiration experiments on tissue cultures in Tyrode with and without chick serum showed the following results.

	Chick serum-Tyrode*	Tyrode alone
	c.mm.	c.mm.
First 15 hrs.	37.3†	24.9
Next 15 hrs.	23.0	8.1

* Controls with serum-Tyrode mixtures without tissue were always observed together with tissue experiments, and corrections made. Since in some of these experiments the presence of dissolved hemoglobin was a disturbing factor, the experiments recorded are only those in which visible hemolysis was absent. Barometric controls, always made, are omitted in the tables.

† Expressed as cubic millimeters of O_2 at N.T.P.

It would thus appear that respiration of tissue in Tyrode with homologous serum continued more actively into the 2nd day than when no serum was present.

The effect of glucose was similarly investigated.

	Tyrode with glucose	Tyrode without glucose
	c.mm.	c.mm.
First 15 hrs.	24.9	11.6
Next 15 hrs.	8.1	1.2

In this experiment it appears that the glucose is an important element in respiration. This is particularly interesting in connection with the fact that not more than 50 per cent of the glucose added to the Tyrode solution appears to be utilized within the 3 day cultivation which is the usual period.[5]

Since it was found that heterologous serum added to chick tissue-Tyrode mixtures neither killed the cells nor inhibited virus and Rickettsia growth, comparative respiratory experiments were done in

[5] We are indebted to Dr. Allan Butler of the Children's Hospital for the quantitative sugar determinations.

which chick serum-Tyrode mixtures were compared with horse serum-Tyrode mixtures.

Oxygen Utilization.—

Period	Chick tissue, chick serum	Chick serum, no tissue	Chick tissue, horse serum	Horse serum, no tissue
	c.mm.	*c.mm.*	*c.mm.*	*c mm.*
24 hrs....................	33.5	3.7	76.9	6.9
Next 15 hrs..............	10.08	Spoiled accidentally	25.2	1.4

It thus appears that the respiration of chick tissue can go on as well in the presence of horse serum as in the presence of chick serum. From a series of runs of chick tissue in horse serum, we have the impression that the horse serum stimulates rather than inhibits oxygen utilization. This, however, is not certain, owing to the occasional quantitative discrepancies in amounts of tissue. More accurate quantitative methods would be necessary to make sure of this.

Comparative respiration experiments were then carried out in which the ordinary amounts of tissue were compared with amounts greater than those favorable for virus or Rickettsia cultivation. These showed that, although the larger amounts of tissue had an increased oxygen uptake in the first 10 hours, roughly approximating the proportion existing between the amounts of tissue added, the oxygen utilization was about the same in the two flasks between the 26th and the 58th hour.

Since tissue heated to 50°C. for 10 minutes is neither viable nor supports Rickettsia or virus growth, such material was also subjected to respiration experiments in Tyrode solution. Controls were made with Tyrode alone. It was found that heated tissues still take up small amounts of oxygen, not, however, amounting to more than about 16 c.mm. in the first 30 hours. Heating to 60°C. practically arrested oxygen utilization. An accident in one of our experiments in which the temperature of the water bath rose to 42°C. for several hours resulted in almost complete arrest of oxygen utilization, indicating the extreme heat sensitiveness of the respiratory mechanism.

Similar experiments, carried out with guinea pig tunica tissue in guinea pig serum, one part, and Tyrode solution, three parts, gave

results similar in principle, though actual amounts of oxygen utilized were proportionately less with guinea pig than with chick tissue. It is particularly important to note that the active oxygen utilization in the guinea pig tissue experiments was practically over after about 30 hours, and usually ceased at the end of 45 hours. 80 to 90 per cent of the oxygen was taken up in the first 30 hours. Thus, since Rickettsia accumulation in these cultures is never apparent before the 6th, 7th or 8th days, it is clear that in the case of these organisms the conditions for growth are established at a time when tissue metabolism has practically come to a standstill. The virus and Rickettsia problems are, therefore, quite distinct in principle, though the Maitland method is used in both cases.

Respiration experiments in which comparisons in oxygen utilization between normal and virus-inoculated tissues were made were not entirely satisfactory because of technical difficulties. In three separate runs, however, such experiments have indicated a suddenly enhanced oxygen utilization in the infected tissue between the 30th and the 40th hour.

Summarizing our respiration experiments, we may say that the period of virus growth corresponds roughly to that during which the tissue is respiring and remains viable. The growth of Rickettsia, on the other hand, seems most active at periods when the tissue has ceased respiring and is either not viable at all or has lost much, possibly all, of its metabolic activity. This fact alone, if any further evidence were necessary, distinguishes the Rickettsia biologically from the virus agents.

DISCUSSION

The purpose of the experiments here recorded was to analyze some of the conditions prevailing in tissue cultures in the hope of thereby gaining insight into the physiology of virus and Rickettsia growth. The Maitland method was selected for analysis because it has become the basic technique for most virus cultivation, and has been successfully modified for the cultivation of Rickettsiae.

In regard to changes of reaction, our experiments indicate that these are determined chiefly by the proportion of tissue amounts to total volume of cultures and the degree to which the formed carbon dioxide

is confined or allowed to escape. When the small amounts of tissue which have been found suitable for virus and Rickettsia cultivation are used in open flasks, the reaction becomes alkaline, changing from an initial pH 7.4 to pH 8.0 and higher by the 3rd day. In similar flasks, tightly stoppered, as is customary in Rickettsia cultivation, where the formed carbon dioxide is retained, the initial reaction of pH 7.4, after a preliminary alkalinity, becomes slightly more acid, averaging pH 6.8 to 7.2 by the 8th or 9th day.

When larger amounts of tissue are used, even in open flasks, the reaction becomes acid, reaching or even surpassing pH 6.0 within a few days. Tissue viability tests have shown that even at reactions as high as pH 9.0 and over, the tissue cells may remain viable, whereas reactions approaching pH 6.0 rapidly injure the cells.

In cotton-stoppered flasks, with the amounts of tissue and Tyrode solution customary for virus cultivation, the presence of virus appears to hasten and increase the development of alkalinity, probably because the tissue is killed and carbon dioxide production ceases.

These observations suggest among other things that the failure of virus growth in the presence of excessive amounts of tissue is due to rapid inactivation of tissue metabolism by the development of acidity. It is not impossible that more rapid and more plentiful virus growth may be obtained with large amounts of tissue if the reaction can be successfully buffered.

Measurements of electromotive potentials in Maitland cultures have consistently indicated that there is a rise of 100 millivolts or more in the course of 72 to 96 hours, after which the potential stabilizes at levels usually in the neighborhood of Eh $+0.420$ (E_1 $+0.082$). There is reasonable agreement between the slowing down of active respiration and potential stabilization.

When Tyrode solution is removed from tissue flasks at the end of 2 or 3 days and used as a menstruum for fresh tissue in a special potential flask, the E_1 values start at the high point and rise still higher in the course of 24 hours.

Potential rises are highest in chick tissue-Tyrode mixtures without serum. The addition of either chick or horse serum diminishes the upward excursion of the potential.

Heating tissue to 50°C. for 10 minutes, a procedure which destroys

the viability of the cells and renders them unsuitable for either virus or Rickettsia cultivation, does not prevent considerable rise of potential. This fact seems of importance, in view of the suggestion of other investigators that potential levels are determining factors in virus cultivation. From our own experiments, we are inclined to assume that while the potential rise is an incidental and possibly important factor, it is not the determining one in virus tissue culture.

In regard to an analysis of the conditions which determine the described changes of potential, we are confronted with a problem involving analysis of the individual enzyme systems, a question which can be approached by the technique we have used, but which must be left to investigators more highly trained than the writers in this type of work.

Our respiration studies have shown that oxygen utilization goes on most actively in the first 40 hours, and then gradually declines up to 70 to 80 hours, when it practically comes to a standstill. Respiration is a little more active in the presence of cultures containing serum than it is in those containing Tyrode solution only. Chick tissue may respire actively in an heterologous serum, *i.e.*, horse serum; and in such heterologous serum cultures, the equine encephalitis virus grows almost as well as in Tyrode solution alone or in chick serum Tyrode cultures. When tissue is heated to 50°C. for 10 minutes, oxygen utilization is not completely stopped, but sinks to a very low level. Temperatures of 42°C. for several hours may materially injure the respiratory mechanism.

An experiment in which comparative respiration measurements were made between flasks containing (*a*) fresh tissue and fresh Tyrode, and (*b*) fresh tissue and "old" Tyrode (the latter previously incubated with tissue for 3 days and separated by centrifugation), showed almost equal oxygen uptakes in the course of 65 hours. This tends to indicate that the cessation of respiratory activity is due neither to the exhaustion of nutritional material in the liquid menstruum, nor to the accumulation of metabolites, but is referable to changes taking place in the cells themselves.

It is interesting to note that only about 30 to 40 per cent of the glucose contained in the cultures is used up, yet experiments comparing glucose-free with glucose-containing flasks indicated the importance

of the sugar for respiration. The amount actually used would have required for its consumption two or three times the amount of oxygen utilization recorded in the respirometer measurements. It seems necessary to conclude from this that a part of the glucose is metabolized by some process in which oxygen takes no part. We have performed no anaerobic respiration experiments.

It seems apparent from our experiments that the growth of a representative virus agent in Maitland cultures is roughly parallel to the metabolic activity of the tissue cells in the cultures. The fact that respiration practically ceases about 12 to 24 hours before the maximum virus titre is found in the fluid may be due to the period required for the extrusion of the virus agent from the disintegrating cells. The decline of virus titre habitually observed on the 4th, 5th and 6th days indicates that the virus not only does not multiply when the cells have ceased metabolizing, but that it deteriorates in the presence of cell disintegration. Mere setting of potentials in cell-free media does not appear to promise solution of the problem of virus cultivation.

Although the general methods are alike for *in vitro* cultivation both of many virus agents and of Rickettsiae, the respective physiological conditions determining growth in these cases seem to be quite different. In contrast to the parallelism in time between virus multiplication and tissue viability and activity, the growth of Rickettsiae appears to be delayed until tissue metabolism has stopped, determinable viability is over and the system has come to some sort of equilibrium. We have found that Rickettsiae can be grown on tissue cultures which are not inoculated until after a preliminary incubation of 3 or 4 days. Nevertheless, even in such cultures, the Rickettsiae invade and multiply in the presumably inactive cells. While, therefore, the Rickettsiae, unlike virus agents, do not depend on the vital activities of the cells, they still appear to require conditions established within the inactive cells before cytoplasmic deterioration has made much progress.

SUMMARY

An analysis of some of the physiological factors active in Maitland tissue cultures has been presented in the hope that it may be of some value in clarifying the principles underlying tissue cultures in general.

It has been found that the empirically determined necessity of using relatively small amounts of tissue in such cultures is dependent upon the fact that excessive tissue leads to a rapid change of reaction toward the acid side. Whereas tissue may remain viable in an environment as alkaline as pH 9 and over, viability is rapidly destroyed when the reaction approaches pH 6.

Evidence is presented to indicate that the changes in electrode potentials which take place in Maitland cultures are not, as has been suggested, the determining factors upon which virus multiplication depends, although they may, of course, be incidentally important.

It has been shown that there are fundamental differences between those conditions in Maitland cultures which favor the multiplication of a typical virus and those upon which the growth of the Rickettsiae of typhus fever depends.

The virus which we have studied (equine encephalitis virus, western type) multiplies during the period of active tissue metabolism. The maximum virus titrations are obtained at about the time at which metabolism has come to a standstill. Thereafter the virus not only ceases to increase but rapidly deteriorates. The period of viability of the tissue cells themselves is shortened by several days in the presence of virus multiplication. There is some evidence that a temporary acceleration of oxygen uptake takes place during the time of active virus multiplication. Technical difficulties in controlling such experiments prevent certainty in regard to this point.

In contrast with the conditions determining the growth of a virus agent in the Maitland cultures the multiplication of Rickettsiae does not begin to any determinable extent until after active cell metabolism has either become stabilized or has ceased. The Rickettsiae continue to grow at a time when the cells are no longer viable. It appears likely that these organisms find the most favorable conditions for growth in cells which are no longer metabolically active but in which some delicately heat-susceptible elements have not yet been disturbed. As a consequence of these observations, frozen and preserved embryonic tissues have been successfully used for Rickettsia cultivation. A report on these experiments will be made in a separate communication.

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A QUANTITATIVE THEORY OF THE PRECIPITIN REACTION

V. The Reaction between Crystalline Horse Serum Albumin and Antibody Formed in the Rabbit*

By ELVIN A. KABAT and MICHAEL HEIDELBERGER, Ph.D.

(From the Departments of Biological Chemistry and Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York)

In earlier papers there has been outlined a quantitative theory of the precipitin reaction and its application to hapten-antibody systems in horse and rabbit antisera (1, 2) and to antigen-antibody systems involving a dye protein (3) and crystalline egg albumin (4). It was shown that if the combination of antigen or hapten were considered to take place in a series of bimolecular competing reactions between multivalent antigen and antibody, simple equations expressing in several instances the entire course of the precipitin reaction could be derived from the law of mass action.

These equations were of the type

$$\text{Antibody N precipitated} = 2 \, R \, Sa \, N - \frac{R^2}{A} (Sa \, N)^2 \dots \dots \dots [1]$$

in which R is the ratio of antibody nitrogen to serum albumin[1] nitrogen at a reference point in the equivalence zone, Sa N is the amount of antigen nitrogen or hapten added, and A is the amount of antibody nitrogen precipitated at the reference point. By dividing through by Sa N the equation

$$\frac{\text{Antibody N}}{Sa \, N} \text{ in the precipitate} = 2 \, R - \frac{R^2}{A} Sa \, N \dots \dots \dots [2]$$

---

[1] Referred to throughout as Sa.

is derived. This is the equation of a straight line and permits evaluation of the constants 2R and $\dfrac{R^2}{A}$, hence also A, for any given serum.

An empirical relation obtained (3, 4) by plotting $\dfrac{\text{Antibody N}}{\text{Sa N}}$ in the precipitate against the square root of Sa N added yielded in some instances an even closer approximation to a straight line than that obtained by [2]. The equation of this line is

$$\frac{\text{Antibody N}}{\text{Sa N}} \text{ precipitated} = 3R'' - 2 \sqrt{\frac{(R')^2 \,(\text{Sa N})}{A}} \dots\dots\dots[3]$$

in which $3R''$ is the intercept on the y-axis and $-2\sqrt{\dfrac{(R')^2\,(\text{Sa N})}{A}}$ is the slope of the line, A = the maximum precipitable antibody nitrogen, and R' = the antibody N:Sa N ratio at the maximum. For example, for serum 3.69, if the equation of the best line obtained by plotting the ratios of antibody N: Sa N in the precipitates against the square root of added Sa N be multiplied by Sa N, the resulting equation describing the behavior of the serum is

$$\text{mg. antibody N precipitated} = 22.4 \text{ Sa N} - 45 \,(\text{Sa N})^{1/2}\dots\dots\dots[4]$$

The present investigation of the precipitin reaction between crystal-line horse serum albumin[1] and the homologous antibody formed in the rabbit was undertaken in order to study the mechanism of a precipitin reaction involving a relatively easily purified serum constituent as antigen. It was thought that a quantitative study of this nature might serve as a starting point for the investigation of chemical and immunological relationships between corresponding proteins of different species. Taylor, Adair, and Adair (5) have also made observations on this reaction and found a zone in which both components appeared in the supernatant. This was ascribed to the presence of several components in the crystalline serum albumin, one of which was later shown by Goldsworthy and Rudd (6) to be globulin.

In the present work comparatively small injections of serum albumin were given and antisera were obtained which were relatively free from antiglobulin. In these sera neither antigen nor antibody could be detected in equivalence zone supernatants, as in the systems previously studied. An antiserum like those studied by Taylor, Adair, and Adair (5) was obtained from a rabbit which had been

subjected to prolonged immunization and it was shown that anti-globulin was present in large amounts.

A preparation of R-salt-azo-biphenyl-azo-crystalline serum albumin was also prepared according to (7). The reactions of this dye protein with homologous antisera and antisera to crystalline serum albumin are also included.

EXPERIMENTAL

The crystalline serum albumin used in this communication was prepared according to (8) and was crystallized three times. For injection the material was precipitated with alum by adding 1 per cent alum and partially neutralizing with very dilute NaOH until precipitation was at a maximum. The suspensions were adjusted to a concentration of about 0.8 mg. of serum albumin per ml. in saline containing 1:10,000 merthiolate.[2] Rabbits were given intravenous injections of the suspension four times a week for 4 to 6 weeks, using a total of 20 to 40 mg. of protein per rabbit. Sera obtained after a second course were usually of higher antibody content. Appreciable amounts of antiglobulin were found only in the later bleedings of rabbit 3.68, which received 229 mg. of Sa.

Quantitative precipitin determinations were carried out as described in previous papers of the series. Nitrogen estimations on the washed specific precipitates were made by the micro Kjeldahl method. As noted in (4), it was occasionally necessary to centrifuge supernatants a second time, especially in the equivalence zone in which the precipitate tends to be loosely packed.

As in (4), it was assumed that supernatants which failed to react with anti-Sa serum in the excess antibody region and equivalence zone actually contained no Sa, since the test for Sa with homologous antibody is extremely delicate, and any soluble Sa-A compound formed would have to be extremely slightly dissociated to escape detection. Moreover, when soluble compounds are present, as in the inhibition zone, they are precipitated when fresh antibody is added. In the region of antibody excess and in the equivalence zone, therefore, N was calculated by subtracting the Sa N added from the total N found in the precipitate.

In Table I are given the effect of temperature and volume changes on the amount of specifically precipitable nitrogen. The solubility of the Sa specific precipitate is about 0.006 mg. N per ml., a value close to that of 0.005 mg. N per ml. found for the solubility of the egg albumin specific precipitate (4). Only very slightly more N was precipitated at 0°C. than at 37°.

Table II shows the result of adding increasing amounts of Sa to a constant volume of antiserum and also gives a comparison of the experimentally determined values with those calculated from equations

[2] Kindly presented by the manufacturers, Eli Lilly and Company, Indianapolis.

[1] and [4]. Since inhibition occurred with small increments of Sa after the maximum had been reached, it was not feasible to derive equations describing the limited region of maximum antibody precipitation.

In the region of slight antigen excess, Sa was determined by adding an aliquot portion of the supernatant to a measured volume of a calibrated serum, estimating the total N precipitated, and reading off the corresponding amount of Sa N from the total N curve of the

TABLE I

Influence of Temperature and Volume on Nitrogen Precipitated by Serum Albumin from 1.0 Ml. of Antisera

Serum No.	Sa N added	Nitrogen precipitated at						Tests on supernatant
		37°			0°			
		2 ml.	2.5 ml.	8 ml.	2.0 ml.	2.5 ml.	8 ml.	
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	
3.69₂	0.05				0.674		0.628	Excess A
	0.25				0.642		0.584	Excess Sa
3.85₁	0.03		0.378			0.402		Excess A
	0.04		0.454			0.474		Trace A
	0.15					0.472	0.436	Excess Sa
3.86₁	0.05	0.930*		0.850	0.940†		0.888	Excess A

Including washings the solubility of the specific precipitate appears to be about 0.006 mg. N per ml. at 0°.

* A similar pair of tubes set up for 2 hours at 37° and overnight in the ice box gave 0.946 mg. N.

† A similar pair of tubes washed four times instead of twice with 2.0 ml. of chilled saline per washing gave 0.916 mg. N.

calibrated serum. The values in the second column of Table II were obtained by subtracting the Sa found in the supernatant from the total added.

In the inhibition zone a similar analysis for Sa N is made, but calculation of the amount of Sa N in the supernatant is complicated by the presence of dissolved antibody and the procedure is as follows (4):

Let A = the maximum antibody nitrogen found in the serum used, Sa = the amount of serum albumin nitrogen added, and N = the amount of nitrogen pre-

TABLE II

Addition of Increasing Amounts of Serum Albumin to Constant Volume of Antiserum

Sa N added	Sa N precipitated	Total N precipitated	Antibody N by difference	Ratio antibody N:Sa N in precipitate	Antibody N precipitated, calculated from equation [1]	Antibody N precipitated, calculated from equation [4]	Tests on supernatants
mg.	*mg.*	*mg.*	*mg.*		*mg.*	*mg.*	
		Course 1.	Rabbit 3.69₁.	1.5 ml. serum used			
0.025	Total	0.392	0.367	14.7	0.370	0.377	Excess A
0.038	"	0.554	0.516	13.6	0 515	0 512	" "
0.050	"	0.668	0 618	12.4	0.630	0.616	" "
0.080	"	0.864	0 784	9.8	0.796	0.775	" "
0.094	"	0.912	0.818	8.7	0 820	0.810	" "
0.100	"	0.904	0.804	8.0		0.813	Trace A and Sa*
0.113	"	0 958†	0.845	7.5		0.818	" " " "
0 125	"	1.000	0.875	7.0			" " " "
0.150	0.144	1.010	0.866	6.0			Excess Sa
0.175	0.150	0.998	0.848	5.7			" "

Equation [1]: mg. antibody N pptd. $= 17.0$ Sa N $- 88$ (Sa N)^2
Calculated from equation, Sa N max. $= 0.0966$ mg.
Antibody N max. $= 0.820$ mg.
Equation [4]: mg. antibody N pptd. $= 22.4$ Sa N $- 45$ (Sa N)$^{3/2}$
Calculated from equation, Sa N max. $= 0.110$ mg.
Antibody N max. $= 0.818$ mg.

Sa N added	Sa N precipitated	Total N precipitated	Antibody N by difference	Ratio antibody N:Sa N in precipitate	Antibody N precipitated, calculated from equation [1]	Antibody N precipitated, calculated from equation [4]	Tests on supernatants
		Course 2.	Rabbit 3.69₂.	1.0 ml. serum used			
0.020	Total	0.354	0.334	16.7	0.333	0.341	Excess A
0 040	"	0.604	0 564	14.1	0.561	0.552	" "
0.050	"	0.674	0.624	12.5	0.635	0.623	" "
0.060	"	0.726	0.668	11.1	0.683	0.677	No A or Sa
0.075	"	0.784	0.709	9 5	0 704	0.721	" " " "
0.100	"	0.820	0.720	7.2		0.725	Trace Sa?
0.112(5)	"	0.856	0.743	6 6			No A or Sa
0.125	0 120	0.858	0.738	6.2			Excess Sa

Equation [1]: mg. antibody N pptd. $= 19.3$ Sa N $- 132$ (Sa N)^2
Calculated from equation, Sa N max. $= 0.073$ mg.
Antibody N max. $= 0.705$ mg.
Equation [4]: mg. antibody N pptd. $= 25$ Sa N $- 56$ (Sa N)$^{3/2}$
Calculated from equation, Sa N max. $= 0.089$ mg.
Antibody N max. $= 0.739$ mg.

* ± after centrifuging.
† One determination lost.

TABLE II—*Concluded*

Sa N added	Sa N precipitated	Total N precipitated	Antibody N by difference	Ratio antibody N:Sa N in precipitate	Antibody N precipitated, calculated from equation [1]	Antibody N precipitated, calculated from equation [4]	Tests on supernatants
mg.	mg.	mg.	mg.		mg.	mg.	
		Rabbit 13.29.		1.0 ml. serum‡ used			
0.025	Total	0.362	0.337	13.5	0.329	0.342	Excess A
0.037(5)	"	0.508	0.470	12.5	0.464	0.468	" "
0.050	"	0.624	0.574	11.5	0.580	0.576	" "
0.062(5)	"	0.740	0.677	10.8	0.675	0.669	" "
0.087(5)	"	0.906	0.818	9.4	0.812	0.806	" "
0.112(5)	"	1.008	0.905	8.0		0.894	No A or Sa
0.125	"	1.042	0.917	7.3		0.924	" " " "
0.135	"	1.070	0.935	6.9		0.935	" " " "
0.157(5)	0.152(5)	1.110	0.957	6.3			Excess Sa
0.200	0.158	1.070	0.912	5.8			" "

Equation [1]: mg. antibody N pptd. $= 14.7$ Sa N $- 62$ (Sa N)^2

Calculated from equation, Sa N max. ≈ 0.1086 mg.

Antibody N max. $= 0.871$ mg.

Equation [4]: mg. antibody N pptd. $= 18.7$ Sa N $- 32$ (Sa N)$^{3/2}$

Calculated from equation, Sa N max. ≈ 0.152 mg.

Antibody N max. $= 0.944$ mg.

		Serum M, used for determination of Sa in supernatants, 1.0 ml.					
0.016	Total	0.228					Excess A
0.032	"	0.390					" "
0.048	"	0.496					" "
0.064	"	0.594†					" "
0.080	"	0.650					No A or Sa
0.080§	"	0.646					" " " "

‡ Absorbed with small additions of globulin until traces of globulin were present in excess.

§ 1 mg. R-salt-azo-biphenyl-azo-crystalline egg albumin added. The washed precipitate was colorless and contained no additional nitrogen.

cipitated at the point considered. Then the amount of specific nitrogen (antigen as well as antibody) in the supernatant is given by A + Sa − N, and all of this nitrogen would be precipitated in the analysis of the supernatant for Sa with excess antibody according to the quantitative theory elaborated in (1), and as was actually found in the dye-antidye system (3). The additional assumption is made that the entire precipitate obtained in this analysis is of uniform composition: in other words, that the dissolved Sa-A present can combine with A until

its composition is the same as that of the Sa-A formed by the free Sa present in its reaction with excess antibody.

If N' = the nitrogen precipitated in the analysis of the supernatant and F = the fraction of the supernatant used in the analysis, $N' \div F (A + Sa - N)$ = antibody nitrogen precipitated from the serum used in the analysis. If the curve of antibody N precipitated by Sa from this serum be constructed, the amount of

TABLE III

Composition of Precipitate in Region of Antigen Excess

Sa N added	Total N precipitated	Specific N in supernatant (A + Sa − N)	Fraction analyzed	Total N precipitated in analysis of fraction	Less specific N in fraction analyzed	Corresponding Sa N	Per cent Sa N in precipitate*	Sa N in fraction analyzed	Sa N in entire supernatant	Sa N in precipitate	Antibody N in precipitate	Ratio antibody N:Sa N in precipitate
mg.	mg.	mg.		mg.	mg.	mg.		mg.	mg.	mg.	mg.	
				Serum 3.69₂, maximum antibody N, 0.743 mg. per ml.								
0.150	0.800	0.093	0.50	0.194	0.147	0.010	6.4	0.0124	0.025	0.125	0.675	5.4
0.200	0.726	0.217	0.50	0.486	0.377	0.036	8.7	0.0423	0.085	0.115	0.611	5.3
0.250	0.642	0.351	0.50	0.660	0.484	0.056	10.4	0.0686	0.137	0.113	0.529	4.7
0.300	0.534	0.509	0.25	0.550	0.423	0.040	8.6	0.0473	0.188	0.112	0.422	3.8
				Serum 3.12, maximum antibody N, 0.449 mg. per ml.								
0.128	0.480	0.097	0.50	0.334	0.285	0.027	8.7	0.029	0.058	0.070	0.410	5.9
0.160	0.418	0.191	0.50	0.482	0.386	0.043	10.0	0.048	0.096	0.064	0.354	5.5
0.192	0.370	0.271	0.25	0.384	0.316	0.031	8.9	0.034	0.136	0.056	0.314	5.6
0.240	0.312	0.377	0.25	0.478	0.384	0.043	10.1	0.048	0.192	0.048	0.264	5.5

Serum M was used for all analyses for serum 3.69₂ except for the last line, for which serum N was used.

Serum 3.13 was used for all supernatant analyses for serum 3.12.

* 100 times value in column 7 divided by sum of values in columns 6 and 7.

Sa corresponding to this quantity of antibody N may be read off. The percentage of Sa in this portion of the precipitate may then be calculated according to

$$\frac{\text{Sa N found} \times 100}{\text{Sa N found} + \text{antibody N found}} = \text{per cent Sa N.}$$

Since it was assumed that the entire precipitate contains this proportion of Sa, $N' \times$ per cent Sa N thus found $\div F$ = Sa N in total supernatant, and Sa N originally added minus this value = Sa N in the original precipitate.

The ratios of antibody N to Sa N in the first portion of the inhibition zone are shown in the last column of Table III, the calculations being those described above. This method was not applicable to the region

TABLE IV

Serial Addition of Serum Albumin to Various Antisera, Calculated to Original Volume

Total Sa N added	Total N precipitated	Total antibody N precipitated	Ratio antibody N: Sa N in precipitate	Antibody N, calculated from equation [1]
mg.	mg.	mg.		mg.
Serum 3.85; 10.0 ml. used				
Equation [1]: mg. antibody N pptd. $= 17.3$ Sa N $- 170$ (Sa N)^2 for 1.5 ml. serum				
0.020	0.424	0.404	20.2	
0 041		0.748	18.3	
0.063		1.107	17.6	
0.086		1.454	16 9	1.30
Serum 3.11; 10.0 ml. used				
Equation [1]: mg. antibody N pptd. $= 12.4$ Sa N $- 76$ (Sa N)^2 for 1.0 ml. serum				
0.080	1.180	1.100	13.8	
0.164		2.012	12.3	
0.252		2.817	11.2	
0.345		3.540	10.3	3.37
0.442		4 179	9.5	3.99
Serum 3.69$_1$; 5.0 ml. used				
Equation [1]: mg. antibody N pptd. $= 19.4$ Sa N $- 78$ (Sa N)^2 for 1.0 ml. serum				
0.050	1.138	1.088	21.8	
0.105		2.102	20.0	
0.166		3.131	18.9	
0.233		4.118	17.7	3.68
0.306		5.023	16.4	4.47
0.387		5.771	14.9	5.17
0.476		6.12	Excess Sa	

5.0 ml. 0.476 supernatant + 1.0 ml.
serum 3.69$_3$ + 0.215 mg. Sa N........ 1.788

1.0 ml. 3.69$_3$ + 0.215 mg. Sa N..... 1.624

Difference...................... 0.164; calculated to original volume =
0.32 mg. N

Total antibody N: recovered 6.44 mg.; present originally 6.60 mg. Recovery,
97.6 per cent.

of marked inhibition since the experimental error is multiplied many times because of the small fraction of supernatant required for analysis with the relatively weak sera available.

Table IV shows the results of serial removal of the antibody calcu-

lated to the original volume in each step after the first. A relatively large quantity of each serum was treated repeatedly with a small fraction of the amount of Sa necessary to remove the antibody. In all three sera, the Danysz phenomenon is quite definite, as can be seen by comparison of the third and fifth columns of the table. It was also observed in serum 3.69_2 that all of the antibody present could not be removed in a serial experiment. However, this residual antibody could be recovered by adding the supernatant to a mixture of fresh serum and Sa. A recovery of 97.6 per cent of the total was obtained, a value well within the limit of experimental error.

Table V indicates that removal of one-half of the antibody from a serum results in a different equation from that of the original serum diluted to the same antibody content as the absorbed serum.

Table VI is a compilation of the antibody N to Sa N ratios in the equivalence zones of the sera studied. The equivalence point ratios are the average of the values found at the antigen and antibody excess ends of the zone.

Table VII shows the results with the second bleeding of rabbit 3.68, which contained a considerable amount of antiglobulin. Although tests on the supernatants with more antibody showed the presence of excess Sa, positive tests were also obtained by the addition of Sa or serum globulin. Similarly, if the serum is precipitated with excess globulin, addition of more globulin or Sa to the supernatant gives definite reactions, showing that antibody is still present.

It was found (Table VIII) that the decreasing antibody content of resting rabbits, previously injected with Sa, was unaffected by the injection of another antigen such as Ea or typhoid vaccine, although a single injection of Sa into the same rabbit produced an immediate drop in antibody content followed by a definite increase.

Preparation of R-Salt-Azo-Biphenyl-Azo-Serum Albumin.[3]—*(Cf. 7.)* 0.46 gm. of benzidine was dissolved in 100 ml. of water containing 1.5 ml. of concentrated HCl. The solution was chilled to 7–8°C., 0.35 gm. of $NaNO_2$ was added, and the mixture stirred until no test for free nitrite was obtained with KI and starch. The solution was poured into 500 ml. of water containing 3 gm. of sodium acetate. 0.87 gm. of R-salt in 100 ml. of water and 20 ml. of 2 N K_2CO_3 were added. The mixture was allowed to stand in ice water for 1 hour before use.

[3] Referred to throughout as DSa (dye serum albumin).

TABLE V

Comparison of Partially Absorbed Serum with Original at Same Antibody Content

Sa N added	Total N precipitated	Antibody N precipitated	Ratio antibody N:Sa N in precipitate
mg.	mg.	mg.	
Serum 3.85 diluted to same antibody content as 3.85B			
0 01	0.166	0.156	15.6
0.02	0.300	0 280	14.0
0.03	0.388	0.358	11.9
Equation [1]: mg. antibody N pptd. $= 17.3$ Sa N $- 170$ (Sa N)^2			
Serum 3.85B after approximately one-half of antibody was removed serially in 4 absorptions			
0 01	0.140	0.130	13.0
0.02	0.238	0.218	10.9
0.03	0.324	0.294	9.8
Equation [1]: mg. antibody N pptd. $= 14.5$ Sa N $- 164$ (Sa N)^2			

TABLE VI

Ratio of Antibody Nitrogen:Serum Albumin Nitrogen in Equivalence Zone

Serum No.	Ratio at antibody excess end of zone	Equivalence point ratio	Ratio at antigen excess end of zone	R calculated from equation [2]	R' calculated from equation [3]
3.13	6.5	(6.3)	(6.1)		
3.12	(9.1)	8.6	8.1		
12.47	7.8	7.1	(6.3)		
13.29	$< 9.4; > 8^*$		(6.5)	7.4	6.2
3.68$_l$	(9)	(7.8)	(6.6)		
3.69$_1$	(8.6)	(7.4)	(6.2)	8.5	7.4
3.69$_2$	$< 12.5; > 11^*$		(6.4)	9.7	8 3
3.85	(11.0)	(8.3)	(5.6)		
Mean.....	8.7	7.6	6.5		

Equivalence point taken as arithmetical mean of the limiting zone ratios.
Values in parentheses calculated from nearest actual determination.
* Not used in calculating mean.

TABLE VII

Behavior of Serum 3.68$_2$. Rabbit Injected with 229 Mg. Serum Albumin

Antigen N added	Total N precipitated	Supernatant plus		
		Sa	Antibody	Sa*
mg.	mg.			
0.04 Sa	0.30	+(++)	+±	+(+++)
0.05 Sa	0.34	+(++)	++	+±(+++)
0.075 Sa	0 35	+(++)	+++	+(+++)
0.42 Sg*	0.41	+++	+++	+++

Readings in parentheses taken after centrifugation.
* Sg = serum globulin.

To 1.03 gm. of crystalline serum albumin in 250 ml. of solution, 10 ml. of 2 N K_2CO_3 were added and 150 ml. of the diazo solution were allowed to run in dropwise with constant stirring. During the addition it was necessary to add 2 N K_2CO_3 several times, a total of 35 ml. being used. At this point addition of diazo solution was stopped since a preliminary test on a small portion showed that an insoluble compound was formed at the equivalent of 200 ml. of diazo solution. After coupling was complete the solution was chilled and acidified with acetic acid to pH 4.7 to precipitate most of the dye protein. The precipitate was suspended in about 150 ml. of cold water and 5 per cent Na_2CO_3 was added until solution was as nearly complete as possible. The solution was centrifuged in the

TABLE VIII

Effect of Heterologous Protein Injection on Antibody Content

Rabbit No.	Date	Antibody N per ml.	
	1935	*mg.*	
3.69₁	Feb. 13	0.58	
3.69₂	Mar. 19	0.74	Allowed to rest
	Apr. 15	0 28	
	" 22	0.18	Then injected intravenously with 10 mg. Ea*
	" 24	0.15	
	" 27	0.15	No precipitate with Ea or anti-Ea
	May 2	0.11	
	" 6	· 0.11	Then injected with 10 mg. Sa
	" 8	0 05	With Ea: −(+ after centrifugation)
	" 13	0.17	0.21 mg. on May 18, 1935; 0.22 on May 22, 1935
3.85₁	Apr. 17	0.72	Allowed to rest
	May 15	0.24	Then injected with typhoid vaccine (4.5 billion organisms)
	" 18	0.14	0.09 mg. on May 22, 1935

* Egg albumin.

cold[4] and the residue discarded. Precipitation with acetic acid left a colorless supernatant which, on neutralization, gave no reaction with anti-Sa. Solution and reprecipitation were repeated twice. In an effort to fractionate so as to obtain a portion which did not react with anti-Sa, the precipitate was dissolved as before in a volume of 130 ml., and 65 ml. of saturated $(NH_4)_2SO_4$ solution were added. The precipitate was centrifuged in the cold (fraction 1). To the supernatant 33 ml. additional $(NH_4)_2SO_4$ solution were added and the precipitate

[4] Using a refrigerated centrifuge manufactured by the International Equipment Company, Boston, Massachusetts.

(fraction 2) was centrifuged off. The supernatant was acidified with acetic acid, yielding fraction 3. When these fractions were redissolved fraction 1 seemed to give the weakest reaction with anti-Sa. It was therefore given two reprecipitations at one-third saturation with $(NH_4)_2SO_4$, after which the supernatant was quite light in color. The precipitate was centrifuged off sharply and suspended several times in cold redistilled acetone until the solvent remained colorless. The residue, which was now insoluble in water, was redissolved in water + K_2CO_3, neutralized, and filtered through a Chamberland L2 filter. Part of the material was precipitated with alum for use in injecting rabbits and part was ultrafiltered in the cold through a parlodion membrane until the filtrate was colorless, in order to remove a considerable amount of dye which was not combined with protein. The ultrafiltered material still precipitated anti-Sa but failed to precipitate at any dilution with an antiserum to R-salt-azo-biphenyl-azo-egg albumin (7). It also failed to inhibit precipitation in the latter serum by the egg albumin dye.

The serum of rabbits immunized with DSa reacted with Sa as well as with DSa and the dye-antidye reaction was not inhibited by R-salt.

An attempt was made to free fraction 2 of reactivity with anti-Sa by chromatographic adsorption, but no fractionation could be observed.

Analytical Data on R-Salt-Azo-Biphenyl-Azo-Crystalline Serum Albumin.—The R-salt-azo-biphenyl-azo grouping, $C_{22}H_{15}O_7N_4S_2$, of formula weight 511.3, contains 12.54 per cent of S; crystalline serum albumin 1.73 per cent of S (9). An analysis by Mr. William Saschek of 8.296 mg. ash-free azo protein gave 1.74 mg. $BaSO_4$, or 2.88 per cent S.

Calculated for 15 disazo groups per molecule...................... 2.84% S
Calculated for 16 disazo groups per molecule...................... 2.91% S

Sixteen tyrosine groups per serum albumin molecule would give a tyrosine content of 4.3 per cent as compared with the value of 4.7 per cent found by the Folin-Marenzi method (10), indicating approximately one disazo group coupled with each tyrosine group present, just as in the case of the egg albumin dye studied (3).

In determining dye N in the precipitate in the DSa experiments, the washed specific precipitate was dissolved with a definite amount of alkali (3), made up to a known volume, and compared in a colorimeter with a DSa solution of known N content to which a corresponding amount of alkali had been added. After the readings had been made the contents of the colorimeter cup were quantitatively transferred to a micro Kjehldahl flask and analyzed for total N. Antibody N was calculated by subtracting the dye N from the total N found.

Table IX shows the result of adding increasing amounts of DSa N and Sa N to a DSa antiserum. It will be seen that Sa failed to remove all of the antibody and that more could be removed from the supernatant by DSa. Both in the unabsorbed serum and in the serum

TABLE IX

Antibody Nitrogen Precipitated from Antidye Serum by R-Salt-Azo-Biphenyl-Azo-Serum Albumin and by Serum Albumin

Antigen N added	3.0 ml. antiserum 4.35 to R-salt-azo-biphenyl-azo-serum albumin											3.0 ml. 4.35 absorbed with Sa					
	Antigen N in precipitate given by Sa	Per cent dye N precipitated	Total N precipitated	Antibody N precipitated	Ratio antibody N:DSa in precipitate	Tests on supernatants	Antigen N in precipitate given by Sa	Total N precipitated	Antibody N precipitated	Ratio antibody N:N.Sa in precipitate	Tests on supernatants	Antigen N in precipitate given by DSa	Per cent dye N precipitated	Total N precipitated	Antibody N precipitated	Ratio antibody N:DSa	Tests on supernatants
mg.	mg.		mg.	mg.			mg.	mg.	mg.			mg.		mg.	mg.		
0.009(3)	0.025	89	0.340	0.315	12.6	Excess A						0.008	82	0.092	0.084	10.5	Excess A
0.019(6)	0.032	86	0.430	0.398	12.4	"						0.016	82	0.176	0.160	10.0	No A or DSa
0.027							Total 0.234		0.207	7.7	Excess A, no Sa						
0.028	0.047	84	0.554	0.507	10.8	Excess A						0.026	67	0.252	0.226*	8.7	Excess DSa
0.037	0.062	84	0.690	0.628	10.1	"						0.033	67	0.260	0.227*	6.9	"
0.039							Total 0.372		0.318	5.9	Excess A, trace Sa						
0.049																	
0.054																	
0.056																	
0.074																	
0.081	0.068	81	0.726	0.658	9.7	No A or DSa	0.434				Trace A, excess Sa						
0.084																	
0.094	0.086	78	0.820	0.734	8.5	Excess DSa	0.412				No A, excess Sa						
0.108																	
0.111																	

* The figures show that antibody still remained in the absorbed serum. 60 per cent of the residual antibody could be recovered by adding a portion to a mixture of DSa and unabsorbed serum 4.35, but no additional antibody was taken out by a mixture of Sa and anti-Sa.

† Single determination on 1.0 ml. serum, calculated to 3.0 ml.

absorbed with Sa the same percentage of the added dye was found in the precipitate in the region of antibody excess. No evidence of a zone with both antigen and antibody in the supernatant was observed.

Table X gives a comparison of the amount of antibody N precipitated by DSa and Sa from an anti-Sa serum. At the maximum both antigens precipitated the same amount of antibody from the serum, and Fig. 1 shows that the same curve and equation result in both cases by plotting the antibody N or the ratios of antibody N to antigen

TABLE X

Precipitation of Anti-Serum Albumin by Dye Serum Albumin and Serum Albumin

Antigen N added	Antigen N precipitated (DSa N)	Per cent antigen N precipitated	Total N precipitated	Antibody N precipitated	Ratio antibody N:DSa N in precipitate	Tests on supernatants	Antigen N precipitated (Sa N)	Total N precipitated	Antibody N precipitated	Ratio antibody N:Sa N in precipitate	Tests on supernatants
mg.	*mg.*		*mg.*	*mg.*			*mg.*	*mg.*	*mg.*		
						Serum 13.28					
		1.0 ml. used with DSa					1.0 ml. used with Sa				
0.025	0.023	92	0.366	0.343	14.9	Excess A					
0.027							Total 0.478	0.451	16.7		Excess A
0.049	0.039	80	0.620	0.581	14.9	Excess A					
0.054							Total 0.776	0.722	13.4		Excess A
0.074	0.060	81	0.794	0.734	12.2	Excess A					
0.081							Total 0.946*	0.865	10.7		Excess A
0.098	0.088	90	0.898	0.810	9.2	No A or DSa					
0.108							Total 1.046	0.938	8.7		Trace A
0.123	0.105	85	1.020	0.915	8.7	Trace DSa					
0.147	0.124	84	1.122	0.998	8.1	Excess DSa					
0.152							(0.151) 1.138	0.987			Trace Sa

* One determination only.

N in the precipitate against the amount of antigen N in the precipitate.

DISCUSSION

The present quantitative study of the precipitin reaction differs from the systems previously studied (1–4) in that evidence exists that the antigen, crystalline horse serum albumin, consists of several components. Although the protein has been crystallized and shown

to have a uniform molecular weight (11) Soerensen has been able to isolate a number of fractions differing in solubility (12a) and Hewitt has separated fractions of widely different carbohydrate content (12b). If the immunological properties of these fractions differ, antisera produced by injection of the serum albumin should consist of a more complex mixture of antibodies than would be produced by injection of a single antigen.

By the injection of smaller amounts of serum albumin than used by other investigators (5), it was found possible to obtain potent antisera which behaved similarly to those yielded by egg albumin (4) and thus rendered feasible a similar quantitative study.

From Table II it will be seen that the equations [1] to [4] (see introduction) which accounted quantitatively for other precipitating systems are equally applicable to the Sa system. The experimentally determined values for antibody N precipitated are in close agreement with those calculated from equation [1] up to the beginning of the equivalence zone and with values calculated from equation [4] up to the maximum. In the derivation of equations [1] and [2] it was found that the volume factors cancelled and that the composition of the precipitate depended on the proportions in which the components were mixed (1). The data given in Table I indicate that the same applies in the Sa system, subject to a solubility correction similar to that obtaining for the egg albumin system (4). Tests on reaction supernatants showed in most of the sera a wide equivalence zone in which neither Sa nor antibody was present, evidence that the Sa was immunologically homogeneous.

From the above data the reaction between Sa and antibody may also be considered to take place in a series of bimolecular competing reactions between multivalent antigen and antibody before precipitation begins. The first step in the reaction would be

$$A + Sa \rightleftharpoons A \cdot Sa$$
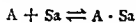
in which A and Sa represent antibody and serum albumin molecules, respectively. This would be followed, for example, in the region of excess antibody by
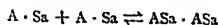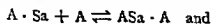
$$A \cdot Sa + A \rightleftharpoons ASa \cdot A \quad \text{and}$$
$$A \cdot Sa + A \cdot Sa \rightleftharpoons ASa \cdot ASa$$

This process would continue, leading to the formation of larger and larger aggregates until these finally precipitated from solution. Such aggregates might be represented two-dimensionally as follows:

```
         :        :        :
         :        :        :
    ····A   ·   Sa   ·   A·······
         ·            ·
         ·            ·
    ····Sa   ·   A   ·   Sa   ·   A·····
              ·        ·
              ·        ·
    ····A   ·   Sa   ·   A   ·   Sa····
    :         ·        ·
    :         ·        ·
         ····A   ·   Sa·····
              :        :
              :        :
              :        :
```
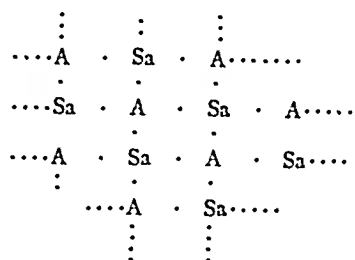
and would resemble those pictured by Marrack (13). It is possible that the insolubility of the precipitate is conditioned not only by the size of the ultimate aggregates but also by a reduction in affinity for water due to a juxtaposition of oppositely charged ionized groupings.

Since inhibition begins with a relatively slight excess of Sa, it was not possible to apply the equations derived for the maximum precipitation zone in the Type III specific polysaccharide-antibody system (1, 2). An attempt to calculate the composition of the precipitate in the immediately succeeding inhibition zone is given in Table III. The method was not applicable near the region of complete inhibition since the weak antisera available necessitated the use of small aliquot fractions in this region, introducing large errors. In one serum, 3.12, the composition of the precipitate remained fairly constant over the region studied, but in another serum a considerable variation in composition was observed, the proportion of Sa increasing with increasing additions of Sa.

The serial experiments shown in Table IV offer further evidence for the presence of only a single antigenic component, since Sa appears in the supernatant only after all the antibody precipitable under these conditions has been removed. In serum 3.69_1, 7 per cent of the antibody was found to be non-precipitable by serial additions of Sa. As in the egg albumin system (4) much of this could be recovered by adding the supernatant to a mixture of antiserum and Sa and determining the additional antibody N precipitated over that given by the Sa and serum alone. A recovery of 97.6 per cent of the total antibody

N could be obtained in this way. The serially non-precipitable antibody may be considered to have a different reactivity from the rest of the antibody, perhaps, as postulated in (4), containing only a single immunologically reactive grouping per molecule. If this were true such antibody could not build up large aggregates by chemical interaction but could attach itself to free Sa groupings on large aggregates already in process of formation. In the serial experiments the Danysz phenomenon is quite definite, as noted in Table IV, and as is required by the theory (cf., for example, (14)).

Table V shows that removal of a portion of the antibody results in a different equation for the residual serum than shown by the original at the same antibody content. This may be taken as additional evidence that antibody to a single substance consists of a mixture of antibodies of different reactivities (cf., for example, (4)).

Table VI indicates the variation in the antibody N:Sa N ratios at either end of the equivalence zone in the sera studied. The values calculated for R and R' in equations [1] and [3] are seen to lie in the equivalence zone. The data show considerable variation in the extent of the equivalence zone in different sera and in different bleedings of the same rabbit, indicating that the zone is characterized by no constant ratio and that no point within the zone would properly be called the equivalence point. A more detailed discussion is given in (4).

Unlike the sera studied in this communication, the anti-Sa sera of Taylor, Adair, and Adair (5) exhibited a marked zone in which both antigen and antibody appeared in the supernatant. This would indicate that the material injected was a mixture of different antigens and that the sera contained a complex mixture of antibodies. The finding of Goldsworthy and Rudd (6) that recrystallized serum albumin contained appreciable amounts of globulin offered an interpretation of these results, for it was possible to show that the serum of a rabbit which had received 229 mg. of crystalline horse serum albumin contained both antialbumin and antiglobulin. In this serum (Table VII) addition of excess Sa (as determined by a test on the supernatant with antibody) left in the supernatant antibody which reacted with serum globulin as well as with Sa (which presumably contained a small amount of globulin). Similarly, addition of excess

globulin left in the supernatant antibody which could be precipitated with either Sa or globulin (which presumably contained a small amount of Sa). The marked zone in which both antigen and antibody appeared in the supernatant is evident from Table VII and resembles the zones observed by Taylor, Adair, and Adair (5). An earlier sample of serum from the same rabbit, after injection of only 41 mg. of Sa, contained only antialbumin and showed an equivalence zone in which neither component appeared in the supernatant. Since Taylor, Adair, and Adair injected about 180 mg. of Sa for each course (15), it is probable that sufficient globulin was injected as impurity to stimulate marked antibody formation.

Table VIII indicates a failure to detect any anamnestic rise in the antibody content of the sera of two resting rabbits injected with heterologous antigens, although in the one instance tested a single injection of the homologous antigen produced a definite increase in antibody content. A similar lack of anamnestic effect has been observed in a rabbit with circulating anti-egg albumin (4).

Table IX summarizes the results obtained with an antiserum to R-salt-azo-biphenyl-azo-crystalline serum albumin (DSa). It will be noted that in the region of excess antibody the percentage of dye N precipitated to dye N added was constant within experimental error. With larger amounts of DSa a sharp equivalence zone appeared, characterized by negative tests with anti-Sa serum, as well. DSa may therefore be considered as a single immunological entity although antisera to DSa contain a mixture of antibodies, as do antisera to other single antigens. Crystalline serum albumin failed to remove all of the antibody N present in the anti-DSa serum and more could be taken out of the supernatant with DSa. The same percentage of DSa reacted in the region of antibody excess with the unabsorbed serum as with the serum absorbed with Sa. After the maximum amount of antibody was removed from the antidye serum first with Sa and then with DSa, a residual portion was still present. This could be removed by a mixture of DSa and unabsorbed DSa antiserum, but was not taken out by an Sa-anti-Sa specific precipitate, indicating that the residual antibody contained antidye groupings rather than anti-Sa groupings.

It is apparent from Table X and Fig. 1 that the same serological

reaction takes place in anti-Sa serum whether or not the Sa molecule is coupled with the dye. Not only do both DSa and Sa remove all of the antibody from an anti-Sa serum, but the same curve or equation results in each case when antibody N or antibody N:antigen N ratios
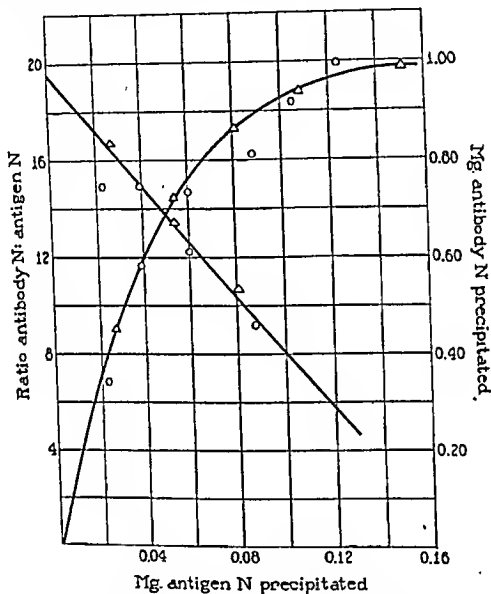
Fig. 1. Precipitation of anti-Sa by DSa and Sa.
O, DSa-anti-Sa reaction.
△, Sa-anti-Sa reaction.

are plotted against the amount of antigen in the precipitate. Similar results were obtained with another anti-Sa serum. That the reactivity with anti-Sa is not entirely due to a possible admixture with Sa is indicated by these data, by the results in anti-DSa serum, and by the behavior of the dye on purification. After removal of all antibody

reactive with Sa from the anti-DSa serum, 82 per cent of the DSa added to the remaining antibody was precipitated, indicating that even if unchanged Sa were assumed to be present in the dye, the amount could not exceed 20 per cent. Actually it must have been much less since part, at least, of the non-precipitable N in the dye was derived from highly colored azo compounds.

Although coupled with the same azo component, the serum albumin dye thus differs markedly in its antigenic properties from the corresponding egg albumin dye (3, 7, 16). While the latter could be fractionated so that it no longer reacted with most anti-egg albumin sera, antisera to the dye exhibited a peculiar cross reactivity with egg albumin (Ea). This reactivity was quantitatively charted (16) and shown to be of a totally different type from either the homologous Ea-anti-Ea reaction or the dye-antidye reaction. In the case of the DSa, however, anti-Sa reacts quantitatively identically with both DSa and Sa, whereas only a fraction of the anti-DSa reacts with Sa. In both dye proteins the number of disazo groups introduced corresponded roughly to the number of tyrosine groups in the antigen, so that the hapten was probably attached to the protein through these groups (cf. Landsteiner (17); (14)), or through histidine. Whatever the points of combination, it is evident that these groupings are only very slightly concerned with the serological specificity of Sa, since their modification produces so slight a change as to be undetectable in anti-Sa serum, even by the sensitive quantitative methods used. The serological specificity of Sa would therefore seem to be determined by groupings of amino acids other than tyrosine and perhaps histidine, while that of Ea would appear to involve molecular groupings including these two amino acids. The structural patterns of the two proteins therefore seem to differ very widely.

Although antisera to the DSa and DEa contained antibodies characteristic of the altered chemical structure, these sera gave only traces of precipitate when tested with the heterologous dye antigen, nor did they show inhibition with R-salt. While such sera have been encountered by Landsteiner (17) they have not hitherto been studied.

With the data now available it is possible to make rough calculations of the equivalent composition of the specific precipitates throughout the reaction range. If one accepts the value of the molecular

weight of Sa as 67,000 (11) and that of antibody (A) in the rabbit as roughly 150,000 (18) the ratio of the weight of A to that of Sa is 2.24. This may also be taken as the ratio of A N to Sa N, since the percentage of N in the two substances is probably not very different.

In the region of extreme antibody excess the average value of 2R, the maximum calculated A N : Sa N ratio, was 13.5 for nine sera. Dividing this by the A : Sa molecular weight ratio, 2.24, gives 6, indicating the equivalent composition of the precipitate at the extreme antibody excess end of the reaction range to be roughly SaA_6. At the antibody excess end of the equivalence zone the mean A N : Sa N ratio was 8.7, corresponding roughly to the equivalent composition SaA_4. At the antigen excess end of the zone, with the ratio 6.5, the composition would be roughly SaA_3. The specific precipitate in the inhibition zone would approximate the empirical composition SaA_2 and the soluble compound or compounds in the inhibition zone could then probably be represented by SaA (*cf.* 19). There would thus be a six-fold range of combining proportions possible between Sa and rabbit antibody.

The above empirical formulas are not offered in the sense that compounds of definite composition are indicated. They are merely approximations of the equivalent composition of the specific precipitate at definite points or regions in the reaction range. The maximum equivalent combining ratio, 6:1, would appear sufficiently small to justify the classical chemical treatment given, even though the formulas derived are not necessarily those of single chemical individuals.

SUMMARY

1. The reaction between crystalline horse serum albumin and homologous antibody in rabbit sera is quantitatively accounted for by expressions similar to those derived from the law of mass action for other immune precipitating systems.

2. The reaction of an azo dye prepared from crystalline serum albumin by coupling with diazotized R-salt-azo benzidine was also studied with homologous antibody and anti-serum albumin.

3. Quantitative data obtained on cross reactions with the two antigens differ markedly from data on the corresponding reactions in the egg albumin system and indicate that tyrosine and perhaps histidine,

while important in determining the serological specificity of egg albumin, have little connection with the specificity of serum albumin.

4. Calculations are made of the equivalent composition of the specific precipitate at various reference points in the reaction range.

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A QUANTITATIVE THEORY OF THE PRECIPITIN REACTION

VI. The Reaction between Mammalian Thyroglobulins and Antibodies to Homologous and Heterologous Preparations*

By HERBERT E. STOKINGER and MICHAEL HEIDELBERGER, Ph.D.

(From the Departments of Biological Chemistry and Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York)

Investigations on the mechanism of thyroid action have emphasized the importance of thyroxine, a crystalline degradation product of the protein, thyroglobulin, which appears to be the actual thyroid hormone. Only limited information is available on the chemical and physical properties of thyroglobulin, on the interrelationships of the thyroglobulins of various animal species, and on the immunological properties of the protein and antisera produced by its injection. Hektoen and his collaborators (1) have made a qualitative comparison and found a greater serological relationship between the mammalian thyroglobulins than between the thyroglobulins of mammals and chickens. On the other hand, absorption experiments revealed distinct differences between certain of the mammalian thyroglobulins. Since a deeper insight into these similarities and disparities might assist in an understanding of the nature of organ specificity it was felt that further study of the problem was warranted, especially since new and more sensitive quantitative methods have become available since the earlier work.

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Preparations of human, hog, beef, and sheep thyroglobulins were made according to (2). Evidence had already been obtained that hog and human thyroglobulins, at least, were of high molecular weight, essentially monodisperse (3), and contained but very small amounts of serum globulin when prepared in this way.

It has previously been shown that typical instances of the precipitin reaction could be quantitatively described by means of equations derived from the law of mass action (4–8). These equations and the significance of their constants, as well as empirical equations which in some instances fitted the data even more closely, were discussed in the preceding paper (8) and are therefore given only in their applied form in the tables in the present paper.

It was thought of interest to test this quantitative theory of the reaction mechanism in the additional case of a protein of far higher molecular weight than had previously been studied.

Taylor, Adair, and Adair (9) have doubted the validity of the assumption made in earlier papers of this series that all of the added antigen is precipitated in the presence of antibody excess. Opportunity for an experimental verification of this assumption was furnished by the use of thyroglobulin, a single antigenic substance containing iodine, an element which could be estimated quantitatively in the specific precipitates.

As a part of this study certain hapten reactions involving diiodotyrosine, thyroxine, and enzymic digests of thyroglobulin are presented, and their relation to thyroid action is discussed.

<center>EXPERIMENTAL</center>

The various thyroglobulins[1] were prepared essentially according to (2). N was taken as 15.8 per cent (2); the serum globulin in two preparations was determined by the quantitative precipitin method (10). Data on the Tg preparations are given in Table I.

Tg suspensions were made and injections carried out as described in (6). Rabbits were injected with 1 to 5 mg. of alum-precipitated Tg four times a week for 4 weeks with a rest period of 1 month between courses. All sera were collected sterilely and preserved with 1:10,000 merthiolate.[2] The quantitative precipitin

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[1] Referred to throughout as Tg.
[2] Kindly presented by the manufacturers, Eli Lilly and Company, Indianapolis.

estimations were carried out as in previous communications by analysis of the washed specific precipitates for nitrogen by the micro Kjeldahl method. All analyses were made in duplicate unless otherwise indicated. To facilitate calculation N values are given to three decimal places although it is realized that the last figure is uncertain.

Owing to the presence of small amounts of serum globulin in the Tg preparations all anti-Tg sera were absorbed with small amounts of serum globulin of the same species as the Tg used for injection, until no further precipitation occurred. Only after this had been done was the Tg-antibody reaction studied. With regard to the proportion of anti-serum globulin found, anti-hog $12E_1$ serum 3.75_8 contained 0.07 mg. of anti-serum globulin N per ml. (analysis on 30 ml.). After absorption 1.07 mg. anti-Tg remained, so that of the total antibody produced during three courses of injection of Tg 6 per cent was due to the serum globulin in

TABLE I

Thyroglobulin Preparations

Preparation No.	Source	Iodine	Serum globulin
		per cent	*per cent*
17 B	Human	0.75	
19 B	"	0.62(5)	
21 B	"	0.31	
13 B₂	Hog	0.58	1.1
13 B₃	"	0.58	2.4
12 E₁	"	0.58	
F B₅	Beef	0.21	
5 A	"	0.68	
F B₄	Sheep	0 34	

the original Tg. Presence of excess serum globulin did not influence the amount of N precipitated by Tg from its antisera. Serum CQ5, absorbed with an adequate amount of hog serum globulin, and at another time with a considerable excess, gave with Tg 0.456 and 0.466 mg. N per ml., respectively. In another instance the excess of globulin was many times that required to inhibit precipitation of its antibody, yet this exerted no effect on the N precipitable by Tg.

The pH of all antisera was ascertained with the glass electrode[3] and, within the limits used, 6.8 to 8.05, no effect of pH change was noted on the amount of N precipitated. Since this is in agreement with previous work on other immune systems (Marrack and Smith (11); (6)), details are omitted.

The solubilities of hog and human Tg specific precipitates at 0°C. have been determined by estimation of N in precipitates washed with varying amounts of

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[3] Courtesy of Mr. F. Rosebury, Department of Biochemistry.

saline, and in precipitates formed after dilution with saline or with normal rabbit serum. The data are summarized in Table II. It will be noted that the solubility of Tg-anti-Tg in 0.9 per cent saline is very small, about 0.003 mg. N per ml. Solubility in normal rabbit serum appeared to be negligible. The solubility of the specific precipitate is thus less than in the other rabbit antibody systems studied (6–8).

TABLE II

Solubility of Thyroglobulin-Antithyroglobulin Precipitates

Volume saline washings	Total N precipitated	Solubility 0°C., N	Tests on supernatants	Volume	Total N precipitated	Solubility 0°C., N	Tests on supernatants
ml.	mg.	mg. per ml.		ml.	mg.	mg. per ml.	
Serum 3.75₂, anti-12E₁, hog 0.5 ml. serum, 0.198 mg. hog Tg N in 0.5 ml.				Serum 3.99₃ anti-19B, human 0.5 ml. serum, 0.158 mg. human Tg N			
4	0.620			4	0 642		
		0.003	Excess A, trace Tg			0.003(5)	Trace A, trace Tg
8	0.608			8	0.628		
Volume							
Serum CQ 4, anti-13B₂, hog 1.0 ml. serum, 0.047 mg. hog Tg N				Serum 3.99₃ 0.5 ml. serum, 0.158 mg. hog Tg N			
1.5	0.148			1	0.252*		
		0.003	Excess A			0.003(5)	Excess A
8	0.128			5	0.238		
Dilution with normal rabbit serum: Serum 3.53₂, anti-13 B₂, hog 1.0 ml. serum, 0.079 mg. hog Tg N				Dilution with normal rabbit serum (compared with above)			
1 5	0.338			4	0.254†		
		0.001	Excess A			0.001	Excess A
8.5	0.332*			8	0.246		

* Single determination.
† Average of three determinations.

Since slightly greater amounts of total N were precipitated at 0°C. for 48 hours than in 2 hours at 37°C. and overnight in the ice box, the former conditions were used unless otherwise indicated; thus anti-hog Tg serum 2.61 gave 0.470 and 0.456 mg. N under the two sets of conditions. At 37°C. no difference in N precipitated was observed in ½ hour or 2 hours, and only additional traces of N were precipitated if the tubes were then allowed to stand overnight in the ice box.

Iodine Content of Specific Precipitates.—In order to ascertain whether or not

the iodine in the specific precipitates was derived entirely from the Tg, antibody-containing globulin was isolated from a typical serum, 3.75₂, anti-12 E₁, by precipitation with 0.85 volume of Na₂SO₄ solution, saturated at 35°C. After dialysis of the precipitate the resulting solution contained 1.56 mg. of N per ml. Duplicate 1.0 ml. samples of this solution were analyzed for I and were entirely negative, as was the saline used in the washings. Iodine determination reagents were I-free. The method used was that of Leipert (12) modified by Dr. G. L. Foster of this department to determine iodine quantities of about 10 γ in the presence of large amounts of protein (20,000 times). The writers wish to express their gratitude for Dr. Foster's aid with these determinations and for permission to use this as yet unpublished method. The above 3.75₂ antibody globulin contained 0.23 mg. of antibody N per ml.

In Table III are summarized the results of the micro iodine determinations on the specific precipitates from four antisera to both human and hog Tg of known N and I content. Points in the equivalence zone, and on both sides of the zone were selected so that the largest possible amounts of iodine could be measured. The same amounts of Tg as were used for precipitation were analyzed for I. After elimination of one determination as out of line, the iodine analyses given in Table III indicate that 96 to 101 per cent of the added Tg is recovered in the specific precipitates up to the first point at which a slight excess of Tg appears. Hooker and Boyd (13) similarly found 90 to 100 per cent of antigen precipitated in the case of hemocyanin. When an excess of Tg is added, a lower proportion of the iodine is, of course, recovered in the precipitate and the relative amount of I (antigen) recovered is that anticipated from the supernatant tests.

Addition of Increasing Amounts of Thyroglobulin to Homologous Antithyroglobulin Sera.—In Table IV are given the quantitative precipitin data obtained by the addition of increasing amounts of human, hog, beef, and sheep Tg to homologous antisera. The course of the reaction is recorded for sera of a single rabbit immunized with the Tg of a single species and given several courses of injections. Analogous data, omitted in the present report, were obtained on many sera, but a summary of data relating to the equivalence zone will be found in Table V for all sera on which a sufficient number of analyses were run. Equations [1] and [4] (*cf.* (8)), are given wherever possible for sera in Table IV and a comparison is made of the precipitated antibody N calculated from these equations with the experimentally

TABLE III

Estimation of Iodine in Thyroglobulin-Antithyroglobulin Precipitates

Tg added (mg.)	I found (gammas)	I calculated (gammas)	I recovered (per cent)	Total N precipitated (mg.)	Tests on supernatants	A/Tg in precipitates, calculated from I and total N
1.0 ml. anti-hog Tg $12E_1$ serum 3.75, hog Tg, 0.58% I, 2 washings, 2 ml. saline each						
1.5	8.8	8.7	101		Excess A	
2.0	11.1	11.6	96		" "	
2.5	14.1	14.5	97	1.26	Trace Tg, excess A	2.3
3.0	16.4	17.4	94		Trace Tg, trace A	
Anti-hog Tg $12E_1$ serum 3.75, hog Tg, 0.58% I						
2.0*	10.9	11.6	9†	1.15	Excess A, trace Tg " "	2.9
3.0†	17.1	17.4	98	1.72	" " " " " "	2.7

Tg added (mg.)	I found (gammas)	I calculated (gammas)	I recovered (per cent)	Total N precipitated (mg.)	Tests on supernatants	A/Tg in precipitates, calculated from I and total N
2.0 ml. anti-human Tg 19B serum 4.00, human Tg, 0.62(5)% I, 2 washings, 2 ml. saline each						
1.25	7.6	7.8	97		Trace Tg, excess A	
1.50	9.3	9.4	99		Trace Tg, trace A	
2.00	11.9	12.5	95	0.63	Excess Tg, trace A	2.3
2.0‡	11.2	12.5	90		Excess Tg, no A	
2.0§	1.0		98		Excess A	
supn't.			(total)			
1.0 ml. anti-human Tg 19B serum 3.99, human Tg, 0.62(5)% I						
2.0‖	12.3	12.5	98	1.36	Trace A, trace Tg	3.4
2.0‡‖	12.3	12.5	98		Excess A	
3 washings with 1.5 ml. saline						
3rd washing: 0.6γ I						

* 2.0 ml. serum.
† 3.0 ml. serum used, single determination in 4 ml. vol.
‡ 1.5 ml. serum.
§ Added to excess antiserum and specific precipitate analyzed.
‖ Single determination.

found values. Agreement between the observed and calculated values was in some cases better and in others less satisfactory than in the examples given.

Several analyses of the specific precipitate were carried out in the region of excess Tg by the method given in (6). Antibody N:Tg N ratios as low as 0.6 and 0.4 were found in the hog Tg system.

Serial Additions of Thyroglobulin to Homologous Antiserum.—Table VI shows typical results of serial additions of small amounts of human and hog Tg to homologous antisera and contains a comparison with the total antibody N which would have been precipitated by single addition at the same Tg N value, calculated to the original volume from Equation [1] as given for the same serum in Table IV. The course of each serial reaction was described with considerable accuracy by the equations given in Table VI.

Heterologous Reactions.—A summary of the proportion of antibody precipitable by heterologous Tg from the various sera is given in Table VII with the age of the sera used. Data on several of the numerous possible cross reactions involving the four Tg's and their antibodies are given in Table VIII. Table IX shows the proportion of antibody precipitable by heterologous Tg remaining after partial precipitation of antiserum with homologous Tg.

Action of Various Related Haptens.—In view of the results reported by Adant and Spehl (14) and Snapper (15) *l*-3,5-diiodotyrosine[4] and *dl*-thyroxine were tested for hapten action in homologous and heterologous human and hog Tg-anti-Tg systems. *l*-3,5-Diiodotyrosine was used in 1:10,000 concentration in 0.9 per cent saline and also as its sodium salt at pH 7.32. *dl*-Thyroxine, due to its extreme insolubility in saline, was used as its disodium salt at pH 10.6.

Addition of these solutions to human and hog anti-Tg sera failed to produce precipitation, and did not inhibit precipitation on subsequent addition of homologous or heterologous Tg. In addition, the alcohol-precipitable material from successive peptic and tryptic[5] digestion of denatured hog Tg, with 41 per cent NH_2 N, gave only negligible precipitates with anti-Tg sera and showed no inhibiting effect.

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[4] Kindly furnished by Dr. G. L. Foster.

[5] Merck's preparation containing polypeptidases; however, digest gave positive biuret test.

The polysaccharide isolated from Tg by alkaline hydrolysis[f] also failed to show hapten action.

DISCUSSION

The immunological property of thyroglobulin which has hitherto been most emphasized is its organ specificity (1). The question of antihormones has, however, recently become one of importance. If, as appears probable, thyroglobulin is the actual hormone of the thyroid gland instead of the crystalline degradation product, thyroxine, thyroglobulin stimulates the production of an antihormone as it readily gives rise to precipitins. The present quantitative study of precipitin reactions involving mammalian thyroglobulins was carried out in the hope of adding to the knowledge of thyroglobulin in both of the above respects and also in order to include in the series of precipitin reactions studied one in which the antigen was of high molecular weight (3).

While the thyroglobulin of no species has yet been obtained in crystalline form, those which have been studied (3) are essentially monodisperse. The thyroglobulins included in the present work behaved as single antigens, in that supernatants in the equivalence zone failed to show the presence of antigen or antibody when tested with fresh antiserum or antigen. Positive reactions in the supernatants from the equivalence zone may be taken as an indication of several components (cf. (8) for a more complete discussion). The preparations used (2) contained small amounts of serum globulin but any disturbing effects of this impurity were eliminated by absorption of all antisera with serum globulin of the same species as the thyroglobulin injected. In agreement with Hektoen and collaborators (1), no immunological relation between serum globulin and thyroglobulin could be detected, a result all the more striking since the thyroglobulins, although so closely related as to show a definite organ specificity, also exhibit marked species differences just as do the serum globulins. In the thyroglobulins these species differences appear to be related to an entirely different molecular configuration than in the case of the serum globulins, since addition of far more serum globulin than re-

[f] To be described in a subsequent communication.

quired to inhibit precipitation of the antiglobulin in the anti-Tg sera failed entirely to reduce the amount of antibody thrown down by Tg.

The solubility data given in Table II indicate a somewhat lower solubility for the Tg-anti-Tg precipitate than was found in this laboratory for other protein-antiprotein precipitates; that is, about 0.003 mg. of N per ml. as against 0.005 mg. in the egg albumin system (6) and 0.006 mg. in the serum albumin system (8). The solubility of the thyroglobulin specific precipitate in normal rabbit serum was only about 0.001 mg. of N per ml. These data also show that the composition of the specific precipitate does not depend on the concentration of antibody at equilibrium, but rather on the proportions in which the reactants are mixed. This would indicate that the considerations which led to the adoption of equations [1] to [4] (see introduction, Paper V (8)) were equally applicable to the Tg system.

Before the analytical data could be fully relied upon it seemed desirable to test the assumption made in previous papers of the series that a single antigen (or hapten) was quantitatively precipitated in the region of excess antibody and the equivalence zone if a test of the supernatant with more antiserum showed no trace of the antigen (or hapten). The reasons for this assumption were fully given in (6) but its validity was questioned by Taylor, Adair, and Adair (9). Since the iodine content of the Tg used was accurately known and anti-Tg had been found to be free from iodine, it was possible to precipitate a given amount of Tg iodine with an excess of antiserum and determine the proportion of the iodine in the washed specific precipitate. As will be seen from Table III, a recovery of 96 to 101 per cent is usually obtained. The above assumption is therefore shown to be justified in the one instance in which a direct test was possible, and this adds an additional reason for its use to those given previously (6).

It appears, therefore, that the ratios given in column 4 of Table IV actually represent the ratio of antibody N to Tg N in the Tg-homologous anti-Tg specific precipitate in the region of excess antibody and in the equivalence zone. Calculations of the composition of the precipitate from the iodine content gave similar ratios of the components, as shown in Table III. When these ratios were plotted against the Tg N precipitated it was usually possible to draw a straight line fairly satisfactorily through the points and thus obtain the constants

for equation [1] for the serum. Usually the points farthest from the line were at the extreme antibody excess end and near the antigen excess end of the equivalence zone. There was thus often a fraction of antibody present in the sera which formed compounds with Tg in which the antibody N:Tg N ratio was greater than 2R, and in such instances the empirical equation [3], in which the ratios were plotted against the square root of the Tg N in the precipitate, often showed better agreement in the region of large antibody excess since this relation covers the formation of compounds of three times the antibody N:Tg N ratio at the maximum (see introduction, Paper V (8)). In Table IV equations [1] and [4], derived from [2] and [3] by multiplying through by Tg N, are given in the instances in which sufficient data were available, and it will be noted that there is fair agreement of the experimentally determined antibody N values with those calculated from one or the other of these relations.   It was evident in most cases, however, that connection of the actual experimental ratio:Tg points led to a curve and not a straight line.

From the above data the reaction between Tg and antibody may also be considered, as a first approximation, to take place in a series of bimolecular competing reactions between multivalent antigen and antibody before precipitation begins. The first step in the reaction would be

$$A + Tg \rightleftharpoons A \cdot Tg$$

followed, for example, in the region of antibody excess, by
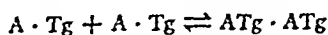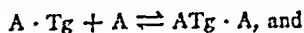
$$A \cdot Tg + A \rightleftharpoons ATg \cdot A, \text{ and}$$

$$A \cdot Tg + A \cdot Tg \rightleftharpoons ATg \cdot ATg$$

This process would continue, leading to the formation of larger and larger aggregates until these finally precipitated from solution, possibly on account of their great size or because of the interaction of polar groups brought into juxtaposition leading to a diminution of affinity for water (cf. also Marrack (16)).

As nearly as could be estimated, R in equations [1] and [4] corresponded fairly closely with the antibody N:Tg N ratio at the antibody excess end of the equivalence zone (Table V). Usually only 80 to 90 per cent of the anti-Tg was precipitated at this point, so that estima-

TABLE IV

Addition of Increasing Amounts of Thyroglobulin to Homologous Antithyroglobulin Rabbit Serum

Tg N added	Total N precipitated	Antibody N by difference	Ratio antibody N:Tg N	Antibody N calculated from equation [1]	Antibody N calculated from equation [4]	Tests on supernatants
mg.	mg.	mg.		mg.	mg.	

Anti-human Tg 17B serum 3.99

Course 1. 1.0 ml. serum, human Tg 17B used

0.079	0.350	0.271	3.4			Excess A
0.158	0.472	(0.314)	(2.0)			Trace A, trace Tg

Course 2. 1.0 ml. serum used

Equation [1]: mg. antibody N pptd. = 6.5 Tg N − 15.0 (Tg N)^2
Max. Tg N, A N = 0.217, 0.704 mg., respectively
Equation [4]: mg. antibody N pptd. = 8.0 Tg N − 10.4 (Tg N)$^{3/2}$
Max. Tg N, A N = 0.263, 0.700 mg., respectively

0.144	0.784	0.640	4.4	0.625	0.582	Excess A
0.216	0.914	0.698	3.2	0.704	0.682	" "
0.288	1.018	0.730	2.5			No A or Tg
0.316	1.043*	0.727	2.3			" " " "
0.364	1.113†	0.749	2.1			" " " "
0.474	1.220†					Excess Tg

Course 3. 1.0 ml. serum used

Equation [1]: mg. antibody N pptd. = 7.7 Tg N − 13.8 (Tg N)^2
Max. Tg N, A N = 0.279, 1.073 mg., respectively
Equation [4]: mg. antibody N pptd. = 11.2 Tg N − 14.2 (Tg N)$^{3/2}$
Max. Tg N, A N = 0.277, 1.029 mg, respectively

0.079	0.686†	0.607	7.7	0.523	0.570	Excess A
0.158	1.024‡	0.866	5.5	0.872	0.864	" "
0.316	1.358‡	(1.042)	(3.3)			Trace A, trace Tg
0.395	1.408†					Excess Tg

Anti-hog Tg 12E₁ serum 3.75

Course 1. 2.0 ml. serum, hog Tg, 13B₂B₃ used

Equation [1]: mg. antibody N pptd. = 4.9 Tg N − 15.2 (Tg N)^2
Max. Tg N, A N = 0.161, 0.395 mg. N, respectively

0.015(8)	0.098	0.082	5.2	0.074		Excess A
0.031(6)	0.170	0.138	4.4	0.140		" "
0.079	0.352	0.273	3.5	0.292		" "
0.158	0.562	(0.404)	(2.6)	(0.394)		Trace A, trace Tg?
0.316	0.804					Excess Tg

* 1.5 ml. 1:1 serum dilution used, calculated to 1.0 ml. whole serum.
† 1.0 ml. 1:1 serum dilution used, calculated to 1.0 ml. whole serum.
‡ One determination only.

TABLE IV—*Concluded*

Tg N added	Total N precipi-tated	Antibody N by difference	Ratio antibody N:Tg N	Antibody N calculated from equation [1]	Antibody N calculated from equation [4]	Tests on supernatants
mg.	*mg.*	*mg.*		*mg.*	*mg.*	

Anti-hog Tg 12E₁ serum 3.75

Course 2.   1.0 ml. serum, hog Tg 12E₁ used

Tg N added	Total N precipi-tated	Antibody N by difference	Ratio antibody N:Tg N	Antibody N calc. eq.[1]	Antibody N calc. eq.[4]	Tests on supernatants
0.158	0.556‡	0.398	2.5			Excess A
0.174	0.590‡	0.416	2.4			" "
0.248	0.720‡	(0.472)	(1.9)			No A, trace Tg?

Course 3.   1.0 ml. serum, hog Tg 12E₁ used.

Equation [1]: mg. antibody N pptd. $= 5.5\, Tg\, N - 9.1\, (Tg\, N)^2$
Max. Tg N, A N $= 0.302, 0.830$ mg. N, respectively
Equation [4]: mg. antibody N pptd. $= 8.2\, Tg\, N - 10.4\, (Tg\, N)^{3/2}$
Max. Tg N, A N $= 0.277, 0.756$ mg. N, respectively

Tg N added	Total N precipi-tated	Antibody N by difference	Ratio antibody N:Tg N	Antibody N calc. eq.[1]	Antibody N calc. eq.[4]	Tests on supernatants
0.032	0.230	0.198	6.2	0.167	0.202	Excess A
0.079	0.508	0.429	5.4	0.378	0.417	" "
0.158	0.784	0.626	4.0	0.642	0.641	" "
0.237	1.018	0.781	3.3	0.793	0.740	" "
0.316	1.168	0.852	2.7			" "
0.395	1.256	(0.861)	(2.2)			Trace A, trace Tg

Anti-sheep Tg FB₄ serum 4.93

Course 1.   1.0 ml. serum, sheep Tg FB₄ used

Tg N added	Total N precipi-tated	Antibody N by difference	Ratio antibody N:Tg N	Antibody N calc. eq.[1]	Antibody N calc. eq.[4]	Tests on supernatants
0.040	0.168	0.128	3.2			Excess A
0.079	0.268	0.189	2.4			Trace A, no Tg
0.249	0.408					Excess Tg

Course 2.   1.0 ml. serum, sheep Tg FB₄ used

Equation [1]: mg. antibody N pptd. $= 6.3\, Tg\, N - 29\, (Tg\, N)^2$
Max. Tg N, A N $= 0.109, 0.342$ mg. N, respectively

Tg N added	Total N precipi-tated	Antibody N by difference	Ratio antibody N:Tg N	Antibody N calc. eq.[1]	Antibody N calc. eq.[4]	Tests on supernatants
0.040	0.262	0.222	5.6	0.206		Excess A
0.079	0.394	0.315	4.0	0.317		Trace A, trace Tg
0.111	0.456‡	0.345	3.1	0.342		" " " "
0.158	0.508	(0.350)	(2.2)			No A, trace Tg
0.332	0.480					Excess Tg

Anti-beef Tg 5A serum 4.83

Course 1.   1.0 ml. serum, beef Tg 5A used

Equation [1]: mg. antibody N pptd. $= 4.5\, Tg\, N - 14\, (Tg\, N)^2$
Max. Tg N, A N $= 0.161, 0.362$ mg., respectively

Tg N added	Total N precipi-tated	Antibody N by difference	Ratio antibody N:Tg N	Antibody N calc. eq.[1]	Antibody N calc. eq.[4]	Tests on supernatants
0.040§	0.213	0.173	4.3	0.158		Excess A
0.079	0.342	0.263	3.3	0.269		" "
0.158	0.498	0.340	2.2	0.361		" "
0.190	0.542‡	0.352	1.9			Trace A?, no Tg
0.237	0.610‡	0.373	1.6			" " " "

§ 2.0 ml. serum used, calculated to 1.0 ml

tion of the total antibody N content of an anti-Tg serum would involve precipitation in the usual way with a slight excess of Tg, estimation of the excess Tg in an aliquot portion of the supernatant by addition to an antiserum calibrated against Tg according to (10), subtraction of the Tg in the total supernatant from the total amount of Tg originally added, and subtraction of the difference from the total N found.

While few analyses of the composition of the Tg-anti-Tg precipitate have been carried out in the region of excess Tg, ratios as low as 0.6 and 0.4 were found for antibody N:Tg N in these analyses. These

TABLE V

Equivalence Zone Ratios of Thyroglobulin Antisera

Serum No.	Antibody N:Tg N ratio at antibody excess end of equivalence zone	Antibody N:Tg N calculated from equation [1]	Antibody N:Tg N ratio at antigen excess end of equivalence zone	Serum No.	Antibody N:Tg N ratio at antibody excess end of equivalence zone	Antibody N:Tg N calculated from equation [1]	Antibody N:Tg N ratio at antigen excess end of equivalence zone
	Human-anti-human				Hog-anti-hog		
3.56₁	> 3.6	4.0		CQ 4₁	< 2.2	2.1	
3.99₁	(3.0)		(2.0)	CQ 5₁	> 2.0	2.1	
3.99₂	(3.0)	3.3	(2.2)	2.40₁	< 1.8	1.9	
3.99₃	> 3.3	3.9	< 3.3	2.61₁	< 2.5	2.7	
				2.61₂		2.1	
	Sheep-anti-sheep			3.75₁	< 3.5	2.5	< 2.6
4.93₁	2.4			3.75₂	< 2.4		< 1.9
4.93₂	(4.0)	3.2	(2.2)	3.75₃	< 2.7	2.8	(2.2)
				3.97₁	2.1	2.1	
	Beef-anti-beef						
4.83₁	< 2.2						

Values in parentheses deduced from nearest actual determination.

instances are an exception to the usual finding that most of the specific precipitate is derived from the antiserum. Ratios smaller than unity were also encountered by Hooker and Boyd (13) in the hemocyanin system and recognized as characteristic of specific precipitates containing antigens of high molecular weight.

It is apparent from Table V that R at the antibody excess end of the equivalence zone was higher in the human Tg-antibody system than in the hog Tg sera. Unfortunately, insufficient data were obtained to fix the other end of the equivalence zone with any certainty, but the

ratios appeared to vary around 2 in both systems. Since it has been shown that the molecular weights of human Tg and hog Tg are the same (3), the apparently broader equivalence zone in the former in-

TABLE VI

Serial Additions of Thyroglobulin to Homologous Antisera, Calculated to Original Volume

Total of successive Tg N additions	Total N precipitated	Total antibody N by difference	Ratio antibody N:Tg N in total precipitate	Ratio antibody N:Tg N in each precipitate	Antibody N calculated from equation [1], Table IV
mg.	mg.	mg.			mg.
7.0 ml. anti-human Tg 17B serum 3.99, total A N 5.11 mg.					
mg. antibody N pptd. = 13.3 Tg N − 8.0 (Tg N)^2					
Max. Tg N, A N = 0.832, 5.53 mg., respectively					
0.031 (6)	0.440	0.408	12.9	12.9	
0.066	0.899	0.833	12.6	12.5	
0.102	1.345	1.243	12.2	11.3	
0.141	1.837	1.696	12.0	11.6	
0.183	2.336	2.153	11.8	11.0	
0.228	2.848	2.620	11.5	10.4	
0.276	3.342	3.066	11.1	9.3	
0.327	3.843	3.516	10.8	8.7	1.90
0.382	4.257	3.875	10.2	6.5	2.17
0.441*	4.611	4.170	9.5	5.0	2.45
Another bleeding, 3.99₃, gave 95% antibody N pptn.					
5.0 ml. 3:1 anti-hog Tg 13B₂ serum CQ 5, total A N 1.55 mg.					
mg. antibody N pptd. = 5.9 Tg N − 10.5 (Tg N)^2					
Max. Tg N, A N = 0.281, 0.826 mg., respectively					
0.023 (7)	0.180	0.156	6.6	6.6	
0.050	0.316	0.266	5.3	4.2	
0.079	0.464	0.385	4.9	4.1	
0.111	0.621	0.510	4.6	4.0	0.420
0.146	0.785	0.639	4.4	3.7	0.540
0.184	0.943†	0.759	4.1	(3 1)	0.665
Another antibody solution, 3.75₂, gave 75% A N pptn.					

The above runs were not made in duplicate.
* Next determination lost, showed slight excess Tg in supernatant.
† Allowed to stand 3 days at 0°C. Trace Tg in supernatant.

stance would seem due to the reactivity of the anti-human Tg with more groupings on the antigen molecule than react with the anti-hog Tg (*cf.* 6).

Perhaps for this reason, which would in some measure determine the range of combining proportions between antigen and antibody, serial additions of human Tg to anti-human Tg serum 3.99$_2$ showed an unusually large Danysz effect (Table VI). All except the final precipitates were characterized in this instance by very high antibody N:Tg N ratios and the equation for the reaction was very different from that obtained with the same serum by addition of increasing amounts of Tg (Table IV). The appearance of Tg in the final supernatants from the serial experiment before all of the antibody N had been precipitated, indicates, as in other systems, the presence in the antiserum of antibodies of varying reactivity toward the antigen. It is probable, also, that the great size of the Tg molecule increases the tendency toward large Danysz effects, owing to the relatively large number of immunologically reactive groupings available. A Danysz effect of similar proportions was obtained with a later bleeding from the same rabbit. On the other hand, two hog Tg-anti-hog Tg serial experiments with much weaker sera gave smaller Danysz effects resembling those observed in other systems (5, 6, 8).

The remaining tables, VII, VIII, and IX, give data relating to the organ specificity of the thyroglobulins. Hektoen and collaborators (1) showed that mammalian thyroglobulins were closely enough related to be termed organ specific, but that they were not necessarily identical. This conclusion is confirmed by the present quantitative studies and extended in a number of respects. In Table VII are given the percentages of cross reacting antibody in many of the sera for which data with homologous Tg are recorded in Tables IV and V. It will be noted that the proportion of cross reacting antibody increases, in general, with prolonged immunization, as would be expected. Moreover, the reciprocal cross reactions appeared to occur to about the same extent, as shown by the human Tg-anti-hog Tg and hog Tg-anti-human Tg data in Tables VII and VIII.

It is shown in Table VII that the cross reactions, also, may be quantitatively expressed by equations [1] to [4], and in this respect the Tg reactions differ radically from two of those previously subjected to quantitative study (17, 18). The antigens in a third cross reaction, crystalline horse serum albumin and R-salt-azo-biphenyl-azo-crystalline horse serum albumin (8) resembled each other even more closely

than do the thyroglobulins, since they behaved identically in antisera to serum albumin.   In the present study 75 to 80 per cent crossing was shown in both directions between sheep and bovine Tg and their anti-sera, the relationship being the closest of those studied.   According to Adant and Spehl (14) sheep and bovine Tg show little crossing, but it is difficult to say whether their conclusion is due, as seems possible

TABLE VII

Approximate Percentage of Cross Reacting Antibody and Age of Antithyroglobulin Sera

Serum No.	Amount of cross reacting antibody	Age of serum	Serum No.	Amount of cross reacting antibody	Age of serum
	per cent	*mos.*		*per cent*	*mos.*
	Hog-anti-human			Human-anti-sheep	
3.56₁	20	1.5	4.93₂	14	3
3.99₁	20	3		Hog-anti-sheep	
3.99₂	36	10	4.93₂	40	2
3.99₃	44	6			
	Human-anti-hog			Sheep-anti-beef	
CQ 4₁	15	2	4.84₁	(75)	2
CQ 5₁	20	2.5		Hog-anti-beef	
2.61₂	30	6	4.84₁	(50)	2
2.64₁	18	9		Human-anti-beef	
3.75₁	12	0.5	4.84₁	15	2
3.75₂	21	6	4.83₁	15	2
	Beef-anti-sheep			Beef-anti-hog	
4.93₁	80	6	4.94₁	45	3
4.93₂	80	2			

Values in parentheses are approximate.

from their data, to inhibition of the cross reaction by the use of excessive amounts of Tg, to reliance on antigen dilution as a measure of antibody reactivity, or to other factors.   Human Tg differs markedly from the others in the series, for in most of the sera obtained after short courses of injections crossing was less than 20 per cent in either direction.   The quantitative precipitin method therefore gives results corresponding in general to the biological relationship of the animals

TABLE VIII

Addition of Increasing Amounts of Heterologous Thyroglobulin to Antithyroglobulin Rabbit Serum

Tg N added	Total N precipitated	Antibody N by difference	Ratio anti-body N:Tg N	Antibody N calculated from equation [1]	Tests on supernatants
mg.	*mg.*			*mg.*	

Anti-human Tg serum 3.99

Course 2. 1.0 ml. serum, hog Tg 12E₁ used

Equation [1]: mg. antibody N pptd. $= 2.6$ Tg N $- 6.4$ (Tg N)^2

Max. Tg N, A N $= 0.203, 0.264$ mg., respectively

0.040	0.130	0.090	2.3	0 094	Excess A, no Tg
0.079	0.242	0.163	2.1	0.165	" " " "
0.158	0.406	0.248	1.6	0.251	" " " "
0.211	0.472	(0.261)	(1.2)		Trace A, trace Tg

Course 3. 1.0 ml. serum, hog Tg 12E₁ used

Equation [1]: mg. antibody N pptd. $= 3.3$ Tg N $- 6.1$ (Tg N)^2

Max. Tg N, A N $= 0.270, 0.446$ mg., respectively

0.079	0.336	0.257	3.3	0.223	Excess A, no Tg
0.158	0.516*	0.358	2.3	0.370	" " " "
0.237	0.624†	0.387	1.6	0.439	No Tg
0.316	0.780†	(0.464)	(1.5)		Trace Tg
0.632	1.016†				Excess Tg

Anti-sheep Tg serum 4.93

Course 1. 1.0 ml. serum, beef Tg 5A used

Equation [1]: mg. antibody N pptd. $= 2.8$ Tg N $- 10.5$ (Tg N)^2

Max. Tg N, A N $= 0.133, 0.187$ mg., respectively

0.040	0.144	0.104	2.6	0.095	Excess A, no Tg
0.063	0.190	0.127	2.0	0.134	" " " "
0.079	0.224	0.145	1.8	0.155	" " " "
0.158	0.354	(0.196)	(1.2)		No A, trace Tg

Course 2. 1.0 ml. serum, beef Tg 5A used

Equation [1]: mg. antibody N pptd. $= 4.4$ Tg N $- 17$ (Tg N)^2

Max. Tg N, A N $= 0.129, 0.285$ mg., respectively

0.040	0.210	0.170	4.3	0.149	Excess A, no Tg
0.079	0.310	0 231	2.9	0.242	" " " "
0.102	0.380	0.278	2.7	0.272	No A, no Tg
0.158	0.440	(0.282)	(1.8)		No A, trace Tg

* Single determination.

† 0.5 ml. serum, calculated to 1.0 ml.

TABLE VIII—*Concluded*

Tg N added	Total N precipitated	Antibody N by difference	Ratio antibody N:Tg N	Antibody N calculated from equation [1]	Tests on supernatants
mg.	*mg.*	*mg.*		*mg.*	
		Anti-beef Tg serum 4.84			
		Course 1. 1.0 ml. serum, sheep Tg FB₄ used			
0.040	0.132	0.092	2.3		Trace A, no Tg
0.079*	0.178	(0.099)	(1.3)		Trace Tg
0.158	0.170	(inhibition)			
		1.0 ml. serum, hog Tg 12E₁ used			
0.040	0.104	0.064	1.6		Excess A
0.079	0.118	(inhibition)			
		1.0 ml. serum, human Tg 21B used			
0.015(8)	0.041‡	(0.025)	(1.6)		Trace Tg
0.040*	0.042	(inhibition)			
		Anti-hog Tg serum 4.94			
		Course 1. 1.0 ml. serum, beef Tg 5A used			
0.020	0.058‡	0.038	1.9		Excess A, no Tg
0.031(6)	0.072	0.040	1.3		No A, no Tg

‡ 2.0 ml. samples calculated to 1.0 ml.

from which the Tg were derived, and should be a more useful tool in serological studies of such relationships than the inaccurate, qualitative dilution methods customarily employed. Rough measurements of relative precipitate volumes in the cross reactions of mammalian sera were made by Nuttall and Strangeways (19).

It is also evident from Table VIII that 2R in equation [1] is usually considerably lower than in the homologous reaction (Table IV). Since the molecular weights of human Tg and hog Tg, at least, have been shown to be the same (3) the ratio effect may be most simply explained by the assumption that the antibody reacts with fewer immunologically active groupings on the heterologous Tg molecule than on the homologous Tg.

Another aspect of the cross reactions is brought out in Table IX. Several sera were fractionally precipitated by the homologous Tg so that as much antibody N, or somewhat more, was removed than had previously been found to enter into the cross reaction. Analysis of

the remaining antibody for cross reacting antibody N showed that nearly one-half remained, so that the heterologous anti-Tg was by no means confined to the portion of the antibody most reactive with homologous Tg. The figures in column 3 show, however, that the initial precipitate was relatively high in the heterologous anti-Tg.

Since data are available on the molecular weights of thyroglobulin (3) and at least a limited number of antibodies formed in the rabbit (20) it is possible to calculate the empirical composition of the specific precipitate at certain limiting points or regions of the reaction range. In the region of extreme antibody excess, as in the serial experiments

TABLE IX

Partial Precipitation of Antisera with Homologous Thyroglobulin, Followed by Precipitation with Heterologous Thyroglobulin

Homologous Tg N added	Homologous antibody N precipitated	Per cent of total antibody N precipitated	Total cross reacting antibody N in serum	Antibody N precipitated by heterologous Tg from supernatant of homologous precipitation	Per cent of heterologous antibody N precipitated
mg.	*mg.*		*mg.*	*mg.*	
		Anti-human Tg 17B serum 3.56₁, 2.0 ml.			
		Hog Tg used in cross reaction			
0.015(8)	0.126	28	0 097	0.043	44
		Anti-hog Tg 12E₁ serum 3.75₁, 2.0 ml.			
		Human Tg used in cross reaction			
0.015(8)	0.082	17	0 084	0.035	42
		Anti-hog Tg 13B₂ serum 2.61₃, 2.0 ml.			
		Human Tg used in cross reaction			
0.015(8)	0.106	27	0.093	0.041	44

(Table VI) ratios of antibody N to Tg N (or antibody to Tg) as high as 12.9 were encountered. If it is admitted from the work quoted above that the ratio of the molecular weights of antibody to thyroglobulin is approximately 150,000:700,000, or 0.21, the antibody:Tg ratio 12.9 would correspond roughly to the empirical composition Tg A_{60}. Ordinarily, however, the values for 2R, as shown in Table IV, do not exceed 8, so that most of the antibody present in a relatively smaller quantity of serum could not form compounds of higher A content than approximately TgA_{40}. The equivalence zone ratios of about 3 and 2 would then correspond roughly to TgA_{14} and TgA_{10}

while the ratio 0.4 in the region of excess antigen would indicate an empirical composition of about TgA_2. The soluble compound or compounds in the inhibition zone in the region of large Tg excess would then have an equivalent composition between this and TgA. It is, of course, not intended to propose these as the chemical formulas of definite, isolable compounds, but they at least indicate the molecular composition of the precipitate at definite points or regions in the reaction range. The extraordinarily great range of combining proportions indicates, it is believed, a very large number of reactive groupings in or on the large thyroglobulin molecule.

Adant and Spehl (14) and Snapper and Grünbaum (15) found no cross reactions between Tg and antisera to artificial iodoproteins or between iodoproteins and Tg antisera. The latter workers also failed to get inhibition of Tg-anti-Tg precipitation by diiodotyrosine or thyroxine and concluded that these two substances do not exist as such in thyroglobulin. While we have also failed to observe inhibition by these amino acids[7] we do not think Snapper and Grünbaum's conclusion justified. A simple calculation shows that even the large Tg molecule contains at most two or three thyroxine groups, not more than eight to twelve diiodotyrosine units, and much unsubstituted tyrosine as well (21). It has been shown above that Tg contains possibly 40 to 60 immunologically reactive groupings and while the iodinated amino acids are chemically the most distinctive and physiologically the most important, there is no reason for or against their being of any significance in the serological reactions of the protein. Moreover, the failure to effect a change in the reactivity of serum albumin in anti-serum albumin sera by introduction of large arylazo groups into at least a high proportion of the tyrosine molecules present in the antigen (8) indicates that chemical changes in the tyrosine groupings are not necessarily accompanied by pronounced changes in specificity.

SUMMARY

1. Quantitative data for both homologous and heterologous precipitin reactions of human, hog, beef, and sheep thyroglobulins show that

[7] The alcohol-insoluble portion of peptic and tryptic digests of Tg and the polysaccharide isolated from Tg after alkaline hydrolysis failed either to precipitate anti-Tg sera or to inhibit precipitation by Tg.

these reactions have the same mechanism as other instances of the precipitin reaction and may be expressed quantitatively by the same equations derived from the law of mass action.

2. It is shown that all of the added antigen is precipitated in the region of antibody excess and in the equivalence zone, so that in these portions of the reaction range the composition of the specific precipitate may be calculated from the nitrogen precipitated and the amount of antigen nitrogen added.

3. The thyroglobulin-antibody reaction is characterized by low antibody N to antigen N ratios, as would be expected with an antigen of high molecular weight. Molecular ratios varying from 60:1 to 1:1 were calculated for the extremes of the reaction range, indicating a very large number of immunologically reactive groupings on the thyroglobulin molecule.

4. Failure of thyroxine or diiodotyrosine to inhibit specific precipitation was confirmed, but it is shown that this need not mean that these substances do not occur in thyroglobulin, as has been claimed.

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VARIATION IN THE SIZE OF TRANSPLANTS OF THE PROSTATE AND SEMINAL VESICLE IN THE ANTERIOR CHAMBER OF THE EYE

By ROBERT A. MOORE, M.D., H. B. ROSENBLUM, S. H. TOLINS, AND ROBERT H. MELCHIONNA, M.D.

(From the Department of Pathology of the New York Hospital and Cornell University Medical College, New York)

PLATE 3

The transplantation of tissue into the anterior chamber of the eye has been employed as an experimental procedure since 1873, but the increased interest in endocrinology in the past decade has led to greater use of this method. Schochet (1) revived the use of ocular or iridial transplants for a study of endometriosis, and Markee (2) developed methods for continuous study of the tissue in relation to physiological activity. The earlier literature is reviewed by Podleschka and Divorzak (3). Markee and Andersen (4) elaborated quantitative methods for measurement of the size of the transplants and recorded a series of changes with pregnancy. Markee, Pasqualetti and Hinsey (5) applied the method to a study of the effects of transection of the spinal cord on the endometrium. Recently, Buxton (6) and Haterius, Schweizer and Charipper (7) have successfully transplanted the anterior lobe of the pituitary gland into the eye.

Despite a considerable number of published observations upon transplanted female accessory sexual organs in female animals, there is only one observation, that of Heckel and Kretschmer (8), on male accessory sexual organs in male animals. They found that the prostate could be transplanted to the eye in rabbits and that the administration of the anterior pituitary-like hormone of pregnancy urine increased the size of successful transplants.

In the present report, methods of transplantation and variations in the size of seminal vesicular and prostatic transplants under normal conditions are described. In subsequent papers, the effect of parenterally administered hormones and the correlation of the size of the transplant and the blood cholesterol will be given.

Methods

Chinchilla rabbits that weighed about 2000 gm. were used in all experiments except in those with prepuberal animals, which were taken immediately after

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weaning. The diet was uniform and consisted of hay, oats, water and fresh green vegetables. The experiments were conducted over a period of 15 months and although the animal room was not at a constant temperature, no variations attributable to season were noted. The rabbits were in separate cages, but in the same room were other rabbits, both male and female, as well as guinea pigs, rats and mice. If an animal contracted snuffles it was not used for experimental observations. All operations were carried out under ether anesthesia and the usual aseptic conditions.

For transplantation, a small piece of the prostate or seminal vesicle was removed through a midline abdominal incision and placed in warm saline solution while the incision was closed. The sclera of the eye was grasped with a forceps and a small incision made into the anterior chamber at the sclerocorneal junction. With a forceps or small eye spoon, a bit of tissue, not over 3 mm. in diameter, was placed in the anterior chamber at the posterior edge of the iris. No preliminary treatment of the cornea with antiseptics was used and no attempt was made to close the incision. After 2 weeks, about 60 per cent of the transplants were well vascularized and could be used for study (Fig. 1).

Daily determinations of size were made by a photographic method (Fig. 2). The rabbit was placed in a vertically sliding box attached to a counterweight so that the eye could be rapidly aligned with the microscope. A monocular microscope with the stage removed and a portable camera with a side observation tube were placed at right angles to the surface of the eye. A 400 watt carbon arc system with a planoconvex lens and a water filter was placed so that the incident ray struck the eye as nearly at a right angle as possible. With this system, satisfactory photographs were secured in 2 to 4 seconds of exposure, with a ×8 ocular and a ×3.2 achromatic objective. The pictures were enlarged and the outline of the transplant drawn. With a planimeter, the square area was determined and recorded on graphs.

Histological study of the transplants in relation to the abdominal prostate and vesicles and the endocrine glands will be reported in a separate paper. An autopsy has been made on all animals and the transplants have been proven to be viable (Fig. 3).

In Fig. 4 a series of daily photographs of the transplant in one animal illustrates the method used.

In order to determine the limit of error of the method, 19 photographs of one rabbit were taken over a period of 30 minutes. A statistical analysis of the measurements of these photographs was as follows:

No. of observations	19
Sum of observations	7271
Mean of observations	382.7
Sum of squares of observations	2,786,029
Standard deviation	13.4
Coefficient of variation	3.4%

No differences in the behavior of the prostatic and vesicular transplants have been observed.

RESULTS

Variation in the Intact Animal.—As shown in Text-fig. 1, there is a variation that exceeds two and one half times the standard deviation of the method and therefore must be considered significant. No regular recurrence or cycle of this variation was observed, even when it was studied over several months. Further, there is no sustained
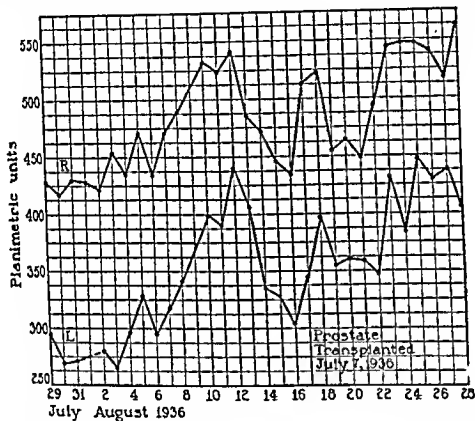
TEXT-FIG. 1. The daily variation in size of bilateral transplants in a normal rabbit.

growth since the transplants are of approximately the same size after 8 months. When transplants are made into both eyes, the variations in the size of the two are directly correlated. This observation indicates that the change in size represents a physiological phenomenon and is related to the general condition of the animal.

In addition to this deviation of from 20 to 40 per cent of mean size, a marked increase of size with eventual return to the original dimensions after 2 months was noted in one animal (Text-fig. 2). This
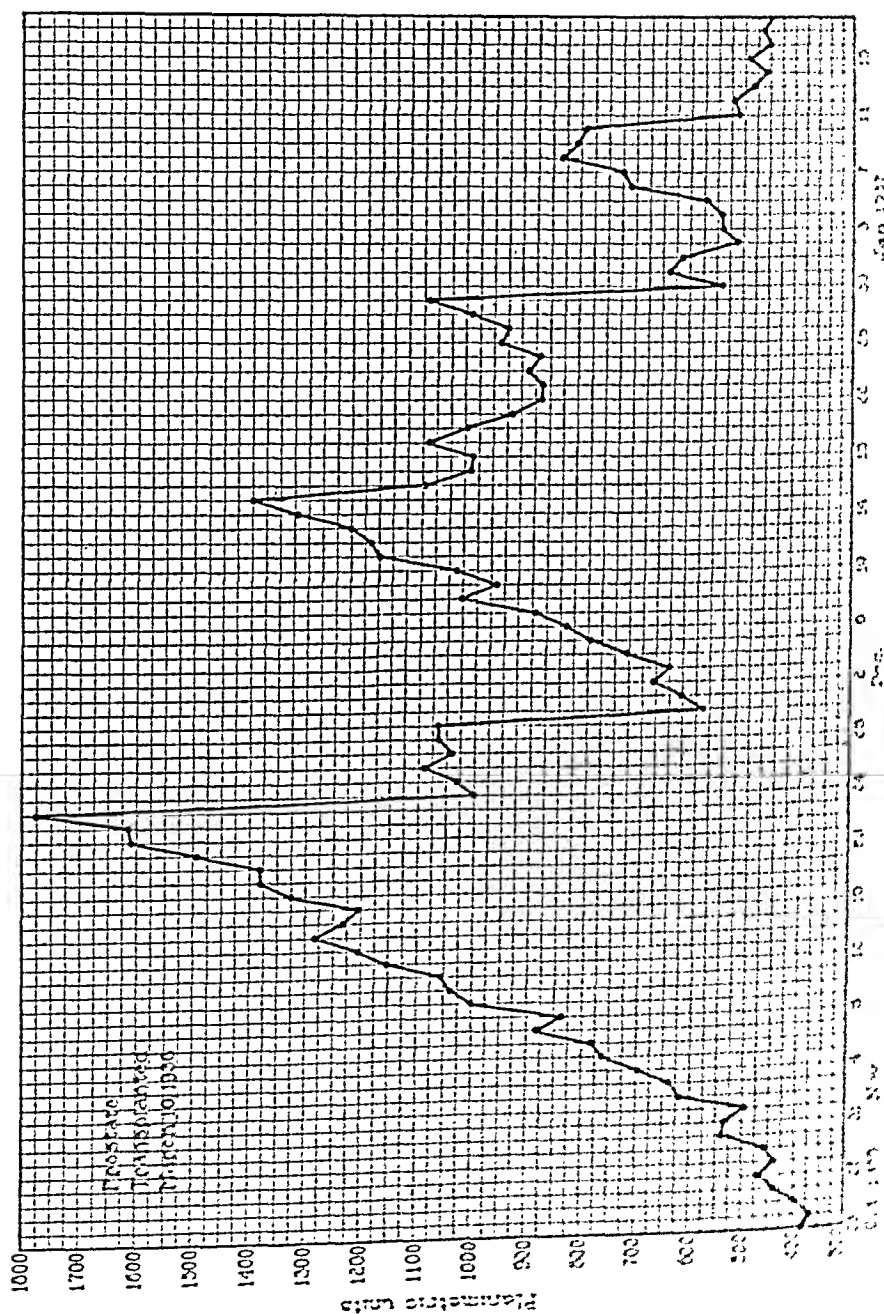
Fig. 2. Marked variation in one animal characterised by gradual increase and precipitous decrease.

period was during the winter months and there was no adequate explanation of the change. The variation in size was characterized by gradual increase and precipitous decrease with a loss of as much as 30 per cent of the total size in 24 hours, and occurred only once in eight rabbits, which were observed for from 6 to 8 months, and in fifteen rabbits which were observed for shorter periods.

The Effect of Castration.—In all mammals the removal of the testes (9) results in a decrease in the size of the prostate and the epithelium
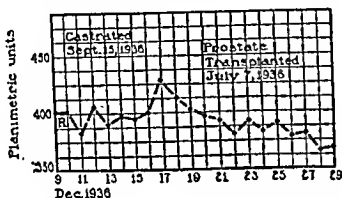
TEXT-FIG. 3. The size from day to day of a transplant in a castrated rabbit.
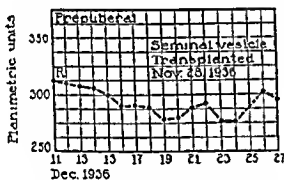
TEXT-FIG. 4. The size from day to day of a transplant in a prepuberal rabbit.

and stroma undergo atrophy. This conclusion is entirely supported by the observations on eye transplants. There is a prompt fall within 48 hours and after 10 days the size is approximately 40 per cent of the precastration average value. During the decrease, which gradually loses velocity, there are temporary periods of increase in size. After several months the irregularity in size from day to day is markedly diminished or absent (Text-fig. 3). This indicates that the testis is responsible for the daily variation.

Transplants in Prepuberal Animals.—In rabbits in which the testes

are intra-abdominal or within the canal, variation is at a minimum (Text-fig. 4). Because the accessory sexual organs of the prepuberal animal are not developed, this observation gives further confirmation to the conclusion that the internal secretion of the adult testis varies from day to day and that this variation is reflected in the size of prostatic transplants.

<div align="center">DISCUSSION</div>

In an evaluation of the results outlined above, it is necessary to bear in mind that the tissue under investigation is in a position where it is deprived of the normal nerve and blood supply. With transplants in one eye, this "out-of-placeness" might be considered as an explanation of the variation, but the close positive correlation of the size of bilateral transplants makes it likely that the variation is the result of a general humoral change. The prompt decrease of size and lack of irregularity from day to day after castration and the minimal irregularity before puberty point directly to the testis as the source of the humoral substance.

In view of the immense literature which has accumulated on the general topic of the endocrinology of the sexual organs, it is remarkable that variation in the male accessory sexual organs has not been previously described. In fact, C. R. Moore (9) states that "hormone production in mammals, not strictly seasonal breeders, appears to be a continuous process. . . ." The reason for this discrepancy is probably to be found in the methods employed for study. The prostate and vesicles in the abdomen are held within a fibrous tissue capsule and surrounded by other organs. In the anterior chamber, the small piece of tissue has no definite capsule and is suspended within a fluid which may be readily absorbed or secreted to allow for changes in size. The method described here is in all likelihood an extremely sensitive indicator of stimulation or depression and will reveal changes not detectable by the usual methods.

The nature of the histological changes in the transplant is under investigation and will be described in a separate communication. When the transplant is largest, the blood vessels are more conspicuous in number and size and no areas within the transplant appear as though acini had filled with secretion. Visible congestion and per-

haps an associated edema are sufficient to account for the changes observed. It cannot be denied, however, that contraction and relaxation of the smooth muscle is also in part responsible. It is not related to local tissue hypersensitiveness, such as that described by Seegal and Seegal (10), since it is a continuous phenomenon and the sections which have been studied show no evidence of inflammation.

SUMMARY

1. A method is described with which the two-dimensional size of a transplant in the anterior chamber of the eye may be measured daily with a high degree of accuracy.

2. The size of transplants of the prostate and seminal vesicles varies from day to day and objective evidence indicates that this variation is related to physiologically active testicular tissue.

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EXPLANATION OF PLATE 3

FIG. 1. Photograph of a rabbit to show the transplant in the eye.

FIG. 2. The apparatus used in these experiments. The details are explained in the text.

FIG. 3. A low power photograph of a transplant of prostate to show the tissue attached by a pedicle to the iris. Hematoxylin and eosin. ×19.

FIG. 4. A series of photographs of a transplant to illustrate the changes in its size. The dates, from left to right, are Jan. 3, 13, 15, 23 and 30, 1937.
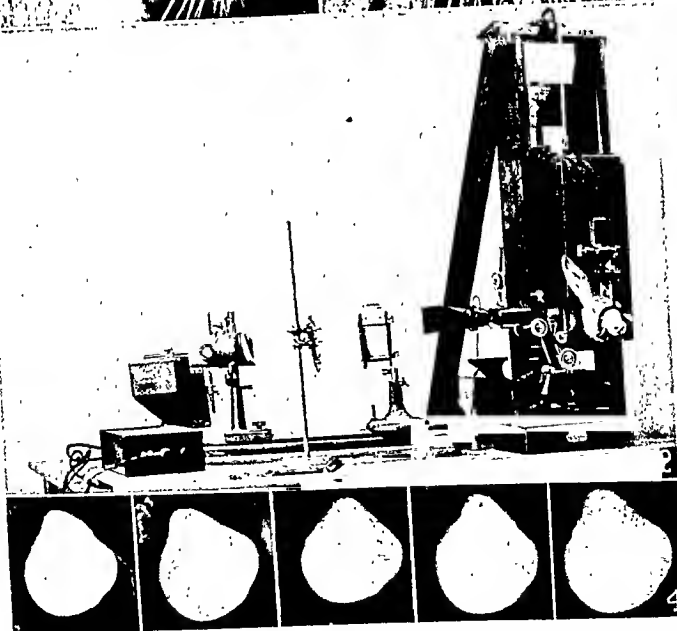
THE PHYSIOLOGICAL RESPONSE OF PROSTATIC AND VESICULAR TRANSPLANTS IN THE ANTERIOR CHAMBER OF THE EYE

By ROBERT A. MOORE, M.D., ROBERT H. MELCHIONNA, M.D., S. H. TOLINS, AND H. B. ROSENBLUM

(From the Department of Pathology of the New York Hospital and Cornell University Medical College, New York)

Markee (1) with Schochet (2) first used transplants of tissue in the eye for physiological observations. With transplants of endometrium in the rabbit, they observed the color changes in the estral cycle and the relation of the vascular changes to muscular contractions (3). Later, Markee and Andersen (4) devised a method for measurement of the size of the transplants by camera lucida drawings and observed a definite series of changes during pregnancy. Litt (5) placed placental tissue in the eyes of pregnant rabbits and observed no effect on the subsequent lactation. After 30 days of growth and establishment of vascularity, there was gradual degeneration and absorption. Goodman (6) with homoiotransplants of immature ovaries in intact and spayed male and female rats observed a number of physiological phenomena. A successful take in a spayed female was accompanied as a rule by regular estral cycles. In the male rat, follicles were formed but corpora lutea did not appear unless extracts of pregnancy urine were administered. The administration of the female sex hormone in the male and female was followed by complete or partial atrophy of the ovarian grafts.

From this incomplete review of the literature, it is clear that, at least with certain organs, transplants in the eye are functioning viable tissues and may be used for physiological observations. With the photographic method which has been described in the preceding paper, it has been possible to follow accurately changes in the size of seminal vesicular and prostatic transplants under the influence of parenterally administered hormones in intact and castrated male rabbits.

Methods

The methods of transplantation and observations of size were identical with the procedure outlined in the preceding paper (7). The hormones used were those

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available on the market. As examples of the gonadotropic substance of pregnancy urine, the trade products of antuitrin-S and follutein were employed. The female sex hormone was represented by theelin and progynon or progynon B. The male sex hormone was employed in the form of oreton or oreton B. The alkaline extract of the anterior lobe of the pituitary was that commercially manufactured by Squibb. Proluton was the example of the hormone of the corpus luteum. The units shown on the graphs were those printed on the labels and in all instances conform to well recognized standards. Differences in the response of seminal vesicular and prostatic transplants have not been observed.

EXPERIMENTAL OBSERVATIONS

Single Injections of the Gonadotropic Substance of Pregnancy Urine in Intact Animals (Text-Fig. 1).—Following three injections each of 100
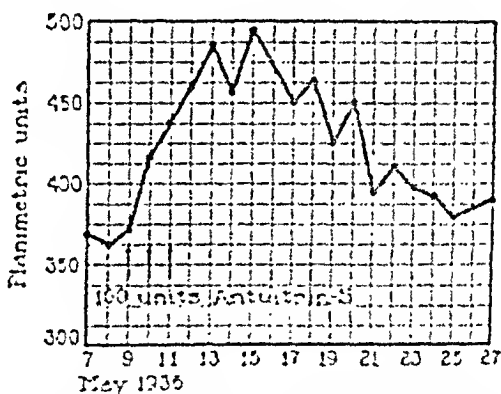
TEXT-FIG. 1. The size of an ocular transplant of the prostate after the injection of the gonadotropic substance of pregnancy urine in an intact rabbit.

rat units of this substance there is a prompt increase in size, manifest within 24 hours of the first injection and reaching a maximum 48 to 96 hours after the third injection. The increase is rapid and continuous but during the subsequent decrease in size there are fluctuations which probably represent superimposed normal fluctuations. The return to the preinjection level is reached in from 10 to 15 days after the injections.

Repeated Injections of the Gonadotropic Substance of Pregnancy Urine in Intact Animals.—With one exception, a second series of injections of similar amounts of this substance fails to induce a response greater than the normal variation, if given within a few months. In one

animal a second series after 3 months gave a typical response but all other attempts have been unsuccessful.

The Gonadotropic Substance of Pregnancy Urine in Castrated Animals (Text-Fig. 2).—In castrated animals this substance in amounts up to 300 rat units is uniformly ineffective and has no influence on the cas-
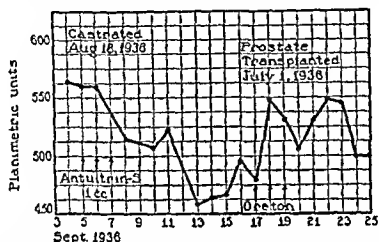
Text-Fig. 2. The effect of the gonadotropic substance of pregnancy urine and of the male sex hormone on the size of an ocular transplant of the prostate in a castrated rabbit.
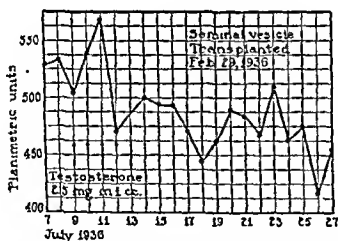
Text-Fig. 3. The size of an ocular transplant of the prostate after an injection of the male sex hormone in an intact rabbit.

tration atrophy. This is true regardless of whether or not the animal has had a previous injection of the same substance.

Male Sex Hormone in Intact Animals (Text-Fig. 3).—The response of intact animals to the male sex hormone is difficult to evaluate because the response is slight and in many instances does not exceed

the observed normal variation of the animal. However, after three injections of 2.5 mg. each, there is always an increase in size on the 1st or 2nd day following the first injection. The increase is rarely sustained and there may be a subsequent fall below the preinjection level.

Male Sex Hormone in Castrated Animals (Text-Fig. 2).—In contrast to the slight response in intact animals, the male sex hormone in castrated animals, in amounts up to 7.5 mg., will within 24 hours decrease the velocity of the castration atrophy and within 3 to 4 days restore the transplant to the precastration size. This restoration lasts for several days and the progressive decrease in size due to castration does not again appear for 10 to 15 days.

The Gonadotropic Substance of Pregnancy Urine and Male Sex Hormone in Intact Animals.—The simultaneous administration of these hormones in the amounts given above results in a prompt increase in size and a slow decrease to the preinjection level, identical with that of the gonadotropic substance alone.

The Gonadotropic Substance of Pregnancy Urine and Male Sex Hormone in Castrated Animals.—In castrated animals which have not received injections of the gonadotropic substance there is a prompt increase in size, analogous in magnitude and velocity to that of the male sex hormone alone.

Alkaline Extract of the Anterior Pituitary Gland in Intact Animals.—This extract (three injections of 1 cc. each) evokes a prompt increase in size within 24 hours after the first injection with a slow decrease over a period of 2 to 3 weeks. The general features of the curve with a smooth increase and irregular decrease are similar to those secured with the gonadotropic substance of pregnancy urine. Repeated injections of the extract give irregular responses but in general less stimulation than the first injection.

Alkaline Extract of the Anterior Pituitary Gland in Castrated Animals.—As in the experiments with the gonadotropic substance of pregnancy urine there is no response to the extract in castrated animals.

Female Sex Hormone in Intact Animals (Text-Fig. 4).—The female sex hormone in three injections each of 2000 international units provokes a conspicuous increase in size, usually slight in 24 hours, but

rapidly increasing after the second and third injections. In contrast to the other hormones, the peak of the reaction is only 24 hours after the last injection. Subsequent decrease in size is always rapid during the first few days but in some animals this is followed by a slower decrease over a period of 10 to 20 days. The form of the curve during
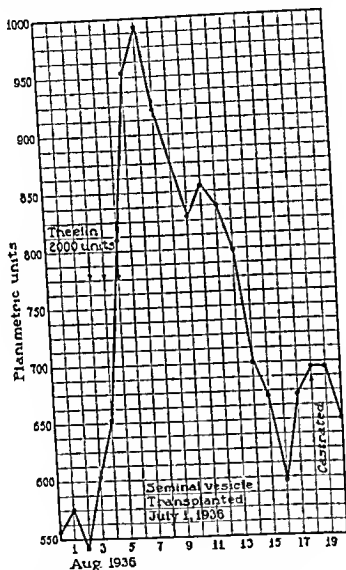
TEXT-FIG. 4. The effect of the female sex hormone on the size of an ocular transplant of the seminal vesicle in an intact rabbit.

the decrease is usually smoother than with the other hormones and shows only occasional and transient periods of slight increase in size. In one of 21 experiments in which this reaction has been tested, there was only a slight increase in size, followed by a decrease below the preinjection level. Both the increase and the decrease were slightly

greater than the normal variation and were probably related to the injection.

Female Sex Hormone in Castrated Animals.—An increase in size, entirely analogous to that in intact animals, results from the injection of the same quantity of this hormone in castrated animals. Repeated injections in both intact and castrated animals result in responses, which in general show no evidence of tolerance or resistance, but the exact percentage increase in size is not always proportional to the units of hormone injected in amounts up to 2000 units.

Male Sex Hormone and Female Sex Hormone in Intact and Castrated Animals. These two hormones in the above amounts injected simul-

TEXT-FIG. 5. Increased vascularity of a transplant after the injection of the female sex hormone. ×25. (Photographs on infrared plates after intravenous injection of trypan blue.)

taneously provoke a response in both intact and castrated animals which in general is similar to that of the female sex hormone alone.

Corpus Luteal Hormone in Intact and Castrated Animals. The injection of this hormone, up to 5 international units, fails to show any change in the size of the transplants either before or after castration.

Vascular Changes with Alterations in Size. Any increase in the size of the transplant in the normal animal or as the result of the injection of a hormone is associated with an increase in the number and size of the visible blood vessels within the graft. In Text-fig. 5, two photographs on infrared sensitized plates show the effect of an injection of the female sex hormone. The pictures were taken after an intra-

venous injection of trypan blue in order to secure a color with maximum sensitivity on the infrared sensitized plates.

Control Observations

When the results described above are reviewed in the light of previous investigations, it is certain that the responses are the result of the action of a hormone. However, there are several possible mechanisms which must be eliminated by control experiments.

Foreign Protein Shock.—The elicitation of a slight to moderate inflammatory reaction in the eye or cornea with prompt healing following foreign protein injections is well known and frequently used in the treatment of corneal lesions. The work of Seegal and Seegal (8) on local tissue hypersensitiveness in the eye also points to the same general type of reaction. In order to eliminate this possibility, animals have been injected with an amount of human serum protein containing nitrogen equivalent to that contained in the injected gonadotropic substance from human pregnancy urine. There was no change in the size of the transplants and therefore this possibility may be eliminated as an explanation of the results.

Hormones from the Same Species.—It may be said that the reactions are in part due to the fact that the hormones have been derived from another animal species and therefore give reactions not analogous to those in a normal animal. In order to eliminate this, implants of eighteen desiccated rabbit pituitary glands were made in one rabbit on 3 successive days and a 25 per cent increase in size resulted.

Focal Inflammatory Reaction.—It is not the purpose of this paper to detail the histological changes in the eye transplants, but sufficient evidence must be presented to eliminate inflammation as the cause of the increase in size. The injection of the female sex hormone results in hypertrophy of the smooth muscle fibers, and marked edema of the connective tissue, but no infiltration with cellular or other elements which would indicate an inflammation in the usual sense.

DISCUSSION

The effects of the injection of hormones on the eye transplants, with the exception of that with the female sex hormone, are entirely in agreement with previous morphological and physiological observa-

tions and need not be discussed in detail. This agreement with other studies indicates that the method is a reliable one and should be useful in other endocrinological investigations.

The lack of response on repeated injections of the gonadotropic substance of pregnancy urine confirms the general thesis of resistance to successive injections of this hormone, but of course throws no further light on the mechanism of the resistance.

The slight response to the male sex hormone in intact, as compared with castrated animals, is in support of the general thesis that a hormone is less effective in an animal that possesses adequate amounts of the functioning tissue elaborating the hormone.

With this method there is no evidence of the synergistic action of the pituitary gonadotropic and testicular hormones which is observed in immature rats and mice. The explanation may lie in the fact that only adult animals were used. Likewise there is no evidence that the two sex hormones are antagonistic. This is an additional fact to be added to those marshalled by C. R. Moore and Price (10) to dispose of the older idea of a fundamental antagonism of the two sexes.

The possibility of an effect of the female sex hormone in the male animal was first shown by Lacassagne (11), who produced carcinoma of the male breast of mice by long continued injections. In the same animals (12), he noted an enlargement of the prostate and microscopically found advanced atypical squamous metaplasia. The diffuse increase in size is conspicuous and may cause urinary obstruction (13). The enlargement has suggested that the female sex hormone may be responsible, at least in part, for benign enlargement of the prostate in man (14), but no convincing proof has yet been given. Recently Zuckerman and Groome (15) have studied a case of benign "hypertrophy" in a dog, in which the histological appearance was identical with the changes induced in dogs by the injection of estrogenic hormones.

In none of the above observations are there described changes within a few days after the hormonal injections analogous to those which are recorded in this paper. Further studies are required to relate the two types of reaction.

SUMMARY

1. With a photographic method for the determination of the size of prostatic and vesicular transplants in the anterior chamber of the eye, it has been possible to follow continuously the response to an injection of a hormone.

2. The results may be briefly summarized as follows: (*a*) One injection of the gonadotropic substance of pregnancy urine produces a moderate increase in size; (*b*) subsequent injections of this same substance for a period of at least 3 months are without effect; (*c*) an alkaline extract of the whole anterior pituitary gland produces a similar increase; (*d*) all pituitary derivatives are ineffective in the castrated animal; (*e*) castration brings about a decrease in size that gradually loses velocity; (*f*) the male sex hormone produces a slight increase in intact, and a variable, at times conspicuous, increase in castrated animals; (*g*) the female sex hormone provokes a conspicuous increase in both intact and castrated animals; (*h*) the hormone of the corpus luteum has no effect; and (*i*) there is no evidence of synergism of the pituitary and male sex hormones nor of antagonism of the male and female sex hormones in adult rabbits.

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THE RELATION OF BLOOD CHOLESTEROL AND THE SIZE OF PROSTATIC AND VESICULAR TRANSPLANTS IN THE ANTERIOR CHAMBER OF THE EYE

By ROBERT A. MOORE, M.D., and J. J. SMITH

(From the Department of Pathology of the New York Hospital and Cornell University Medical College, New York)

The relationship of certain endocrine glands to the blood cholesterol is well established. Denis (1) found that in Graves' disease there was hypocholesterolemia and the reverse in myxedema. Westra and Kunde (2) secured similar results in experimental animals, when the higher values in cretin rabbits were lowered by the feeding of desiccated thyroid.

It has been clearly established by the studies of Slemons and Stander (3), Boyd (4) and others, that there is an increase in the free and total plasma cholesterol in pregnancy. Okey and Boyden (5) reported that the blood cholesterol was conspicuously and consistently lowered during or within a few days before menstruation. The attempt has been made, with doubtful success, to relate these changes in blood cholesterol to the available active estrin in the body.

In view of the conspicuous increase in the size of prostatic and vesicular transplants following injections of estrin in the male rabbit reported in the preceding papers (6, 7), it seemed desirable to investigate the level of the blood cholesterol during the period of the enlargement. Since other hormones also caused enlargement, it was of interest to study these two factors under the influence of the male sex hormone and the anterior pituitary-like substance of pregnancy urine.

Methods

The procedures described in the preceding papers (6, 7) were used for the transplantation and measurement of the grafts in the eyes of male rabbits. Free cholesterol was determined according to the blood lipid methods of Kirk, Page and Van Slyke (8). Briefly, the method consisted in the carbon combustion of the material precipitated by digitonin from the alcohol-ether and petroleum ether soluble extractives of heparinized plasma. The carbon dioxide liberated by wet combustion was collected and determined by manometric methods.

The rabbits were fed a diet of hay, oats and water, and a constant quantity of lettuce was given each day. No food was given for at least 16 hours before the blood samples were taken. Blood was collected from the ear vein into calibrated tubes in order to keep the dilution by the heparin constant. The plasma was removed after centrifugalization and extracted with alcohol and ether.
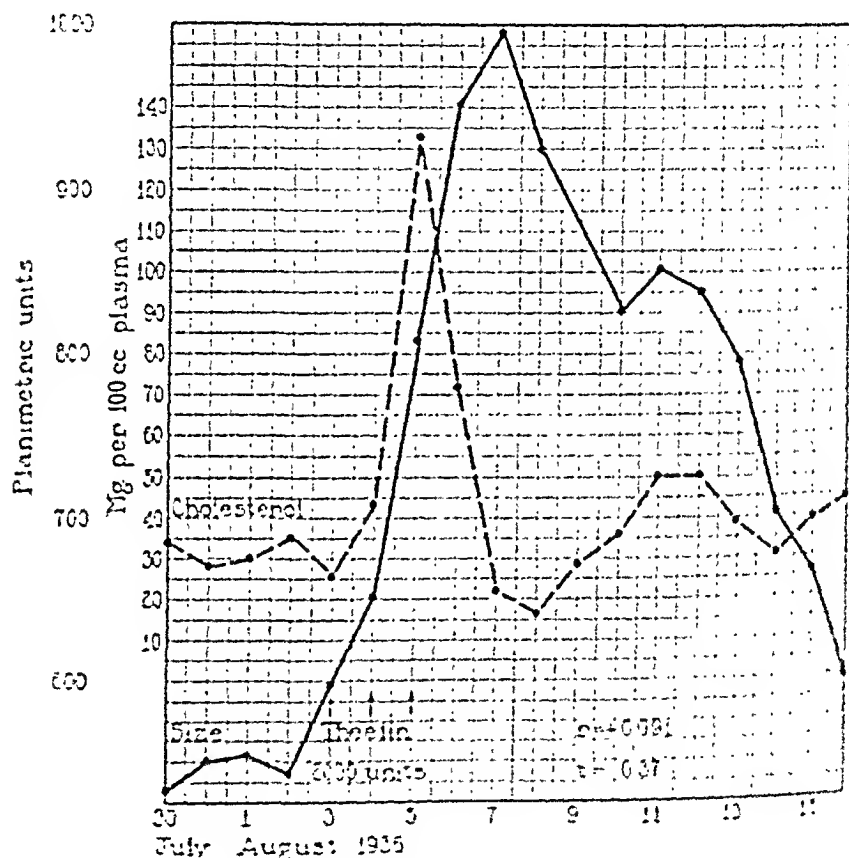
TEXT-FIG. 1. Transplant size and blood cholesterol with the injection of the female sex hormone.

The effect of this daily loss of blood was studied in order to eliminate it as a significant factor in any changes observed. After from 2 to 3 weeks of this experimental procedure, there was an average fall of 25 per cent in the hematocrit value there, but no change in the serum proteins. In animals in which no more injections were given, there was a more rapid return in the body weight and it is improbable that this anemia is of importance in these studies.

Preliminary experiments, in which total lipid carbon was determined, showed no significant correlation between this value and the size of the transplants.

RESULTS

The Female Sex Hormone.—In Text-figs. 1 and 2 are shown a typical response of a vesicular transplant and the blood cholesterol to an
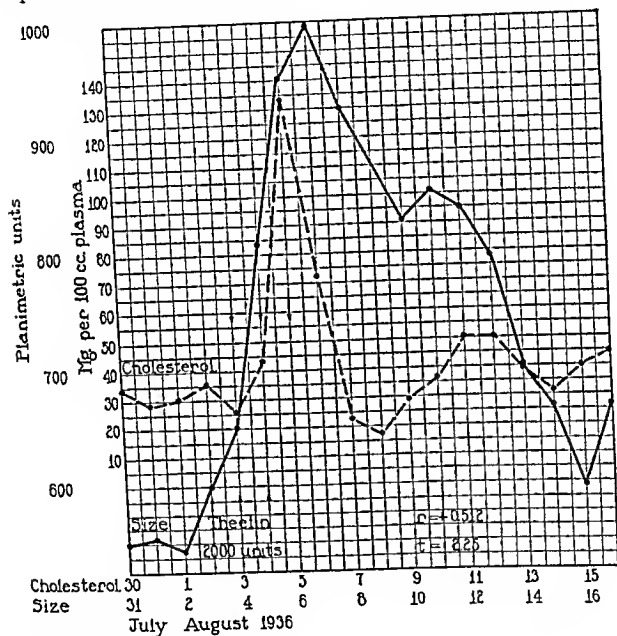
TEXT-FIG. 2. Same as Text-fig. 1, except that the values for the blood cholesterol are advanced 1 day.

injection of the female sex hormone. The results in Text-fig. 2 are arranged so that the determinations of size, shown in Text-fig. 1, are advanced 1 day. There was a prompt increase of the blood cholesterol

level which was followed in 24 to 48 hours by an increase in size of the transplant, as described in the preceding paper. When the values for each day were analyzed (Text-fig. 1), the correlation coefficient was +0.091 with a probable error of 0.37. In sharp contrast was the correlation coefficient of +0.512 with a probable error of 2.25, when the
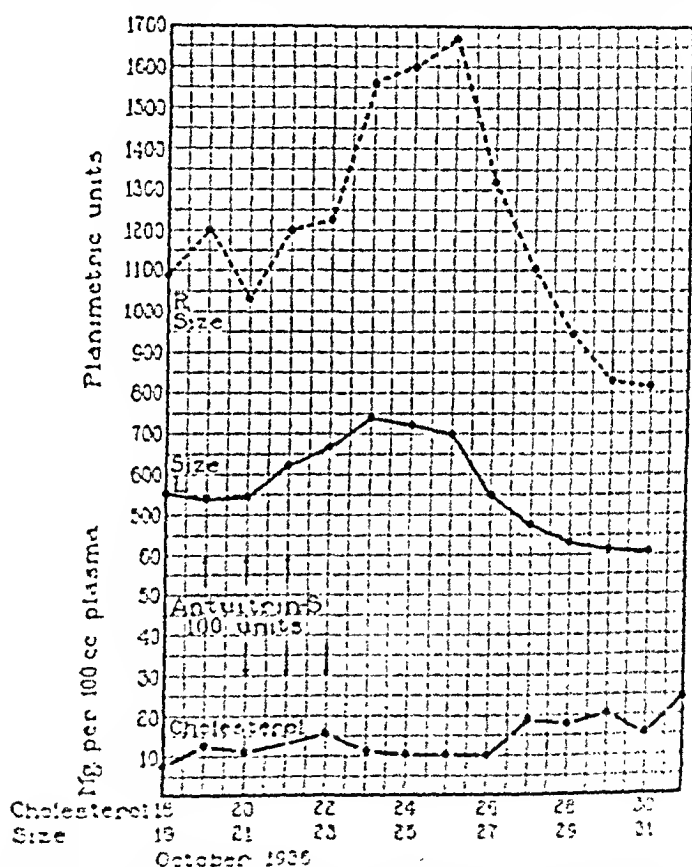
TEXT-FIG. 3. Transplant sizes (right and left eye) and blood cholesterol with the injection of the anterior pituitary-like substance of pregnancy urine.

size was advanced 24 hours (Text-fig. 2). It should be noted that the secondary rise in size during the recovery period was also correlated with an increase of the blood cholesterol. This elevation of cholesterol was also found in the castrated animal when the female sex hormone was injected, although in general it was not as conspicuous:

The Gonadotropic Substance of Pregnancy Urine.—In Text-fig. 3 are the results in an intact animal, which had not received a previous injection. The transplant undergoes the usual response, which has been described, but there was no alteration in the cholesterol values. In the castrated animal neither the transplant size, nor the cholesterol, was significantly affected.

Male Sex Hormone.—In the intact animal (Text-fig. 4), there was a slight increase in the size but *no effect on* the cholesterol in response to the male sex hormone. In the castrated animal, a moderate effect
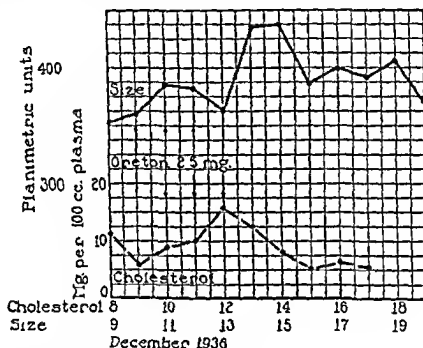
Text-Fig. 4. Transplant size and blood cholesterol with the injection of the male sex hormone.

on the transplant was observed, but no change in the cholesterol values.

Ingestion of Cholesterol.—Two animals were given by stomach tube 0.5 gm. of pure cholesterol dissolved in sunflower oil, on 3 consecutive days. In both, there was an increase in the blood cholesterol comparable to that which results from administration of the female sex hormone, but no significant alteration in the size of the transplant.

DISCUSSION

It is clear that of the three hormones, the male and female sex hormones and the extract of pregnancy urine, which cause significant

changes in the size of vesicular and prostatic transplants in the eye, only one, the female sex hormone, provokes a correlated increase of blood cholesterol. It is also clear that the presence or absence of the testes is of no significance in this correlation.

There are two possible conclusions: first, that the female sex hormone acts on two mechanisms to produce, on the one hand, changes in the prostate, and, on the other hand, alterations in the metabolism of cholesterol; and second, that a single effect in the rabbit is manifested in two different ways. The fact that other hormones produce indistinguishable increases in the size of the transplant but no elevation of cholesterol, indicates that the former conclusion is correct. This is further supported by the observation that increase of blood cholesterol by feeding does not carry with it changes in the transplants.

SUMMARY

In male rabbits, injections of the female sex hormone result in marked increase in the size of ocular transplants of the prostate and seminal vesicles and a correlated elevation of the blood cholesterol.

Other hormones, which cause an increase in the size of the transplants, do not show this correlation.

The ingestion of cholesterol with conspicuous increase of the blood cholesterol, has no effect on the size of the transplants.

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THE CULTURE OF WHOLE ORGANS

II. The Effects of Perfusion on the Thyroid Epithelium

By HARALD OKKELS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 4 AND 5

With the possibility of cultivating whole organs in the Lindbergh apparatus (1) a new field in experimental biology has been opened up. During the last year numerous experiments with culture of the isolated thyroid gland have been carried out in this laboratory. The technique of operation and cultivation has been described in a previous paper (2). The present report deals with the morphological findings in more than a hundred cat and rabbit thyroids which have been cultivated for various lengths of time.

Technical

The animals were etherized and the thyroid gland dissected and inserted into the organ chamber of the Lindbergh apparatus as described by Carrel (2). Either the right lobe was isolated for cultivation, while the left lobe was saved for immediate histological cultivation; or both lobes were dissected and cultivated separately. Generally, the culture medium contained 20 per cent homologous serum diluted with glucose-Tyrode solution; 5 mg. per cent phenol red was added as indicator. In many cases 40 per cent serum was used. As soon as the perfusion was stopped, the glands were removed and fixed for histological examination. Maximov's Zenker-formol solution was used as the standard method for fixation. The material was embedded in paraffin, cut in short series at 5 microns and stained with hematoxylin-eosin, Heidenhain's iron-hematoxylin and sometimes Bensley's and Masson's stains. In a number of cases small pieces were fixed and saturated according to the Ludford modification of the Mann-Kopsch osmic acid technique. The Da Fano silver impregnation has been utilized but did not prove satisfactory.

Effects of Perfusion on the Thyroid Epithelium

During cultivation the thyroid gland does not show appreciable macroscopical changes, provided the circulation is kept at a normal

rate and no accidents occur. In the areolar connective tissue which envelops the gland, a considerable edema may develop due to venous collaterals; but neither does this seem to affect the gland, nor is it accompanied by any edematous state of the thyroid tissue itself.

Microscopically, the gland may remain normal or show areas in which the cells are shrunken, the nuclei pycnotic and the general aspect that of a necrobiotic state of the tissue. Such alterations are not uncommon. They may appear only as small spots, or they may take up a considerable part of the total amount of parenchyma. In the more extensive form, this condition is, however, of exceptional occurrence. We have not been able to trace the cause of the damaged state of small areas of the tissue. It is possible that minute particles or small air bubbles which have been able to pass the filters, are responsible for the appearance of such degenerated spots by causing small emboli. Anyway, the presence of such partial alterations does not seem to influence the morphology of the remaining tissue.

In our attempt to describe the histological and cytological aspect of thyroid glands after cultivation, we have found it convenient to arrange the material in groups: glands cultivated for less than 24 hours; glands cultivated for 24 to 48 hours; glands cultivated for about 1 week; and glands cultivated for more than 1 week.

Cultivation for only a few hours will not bring about variations in the form and size of the thyroid vesicles. The colloid is as a rule not affected, its stainability and density remain unaltered and the number and size of vacuoles are practically the same as in the control glands. The cells in some vesicles may show a very slight swelling, but this is by no means a constant finding. As the structural aspect of the cytoplasm is essentially the same whether the duration of the experiment is 4 or 24 hours, we shall reserve the descriptions of these findings for the next group.

In glands which have been cultivated over periods of between 24 and 48 hours, the form and size of the vesicles is the same as in the control glands. The colloid is of the same density and stainability, and the amount of vacuolization is only slightly increased. This change is, however, not at all constant and it is not present in all vesicles. The vacuolization seems to be less liable to occur in the larger vesicles with dense colloid and flattened cells than in the small or

units. The epithelium in a cultivated gland is generally slightly higher than in the control. But again, this change is not taking place uniformly throughout the parenchyma. And again, the smaller vesicles, and especially those in the central part of the gland, are more liable to show the reaction than are the larger ones. This cellular swelling is an increase in volume which is different from the edematous state which may precede cellular degeneration. It is much more like the initial swelling of the thyroid cells which is observed during the first stages of experimentally increased secretory activity. Not all the thyroid cells along the periphery of any given vesicle show the

50μ
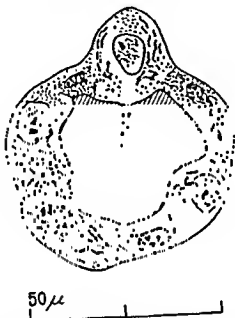
TEXT-FIG. 1. Vesicle from rabbit thyroid gland, perfused for 24 hours with 40 per cent rabbit serum in glucose-Tyrode solution, showing considerable swelling of a single epithelial cell. Osmic impregnation; Heidenhain's iron-hematoxylin stain.

same degree of swelling. Most commonly, only a few cells wide apart will react; in vesicles where most of the cells are cuboidal or flat, the contrast between the swollen clear cells and their more deeply stained neigbbors may be very striking (Text-fig. 1).

The mitochondria are either of the filamentous or of the short rodlike type (Fig. 6). They are mostly well conserved and normally stainable in the preparations from glands cultivated for 48 hours. The distribution and size correspond well with what is found in the uncultivated gland from the same animal. The Golgi apparatus of the thyroid cells in glands perfused from 24 to 48 bours is reticular in

shape and its localization and general structure is the same as in the control glands (Fig. 3). It is interesting to note that precisely in the type of swollen cells mentioned above, the Golgi apparatus is expanded and partially disintegrated in a manner which closely resembles that which is observed during the first phase of stimulated activity of the thyroid cells. The reticular material is much less than usual and small fragments of the filaments—not granules—are floating in the clear swollen cytoplasm of the supranuclear region of the cell.

The blood vessels and the mesenchyme do not show any sure signs of alterations due to perfusion during 1 or 2 days.

Cultivation of the thyroid gland for about 1 week need not affect the general histology of the gland. The size of the vesicles may show a tendency to increase. The colloid may show a somewhat higher degree of vacuolization and, in some vesicles, a different stainability compared with the lobe fixed immediately after the death of the animal. Generally, the gland is in a morphologically good condition. Cytological examination reveals, however, that structural changes have actually taken place during the experiment. The mitochondria are mostly granular or indistinct, and the Golgi apparatus does not show a filamentous or reticular shape any longer; instead, an accumulation of osmiophile droplets is seen to coincide with the Golgi zone of the cytoplasm. It seems as if the finer cytoplasmatic structures gradually undergo degeneration during the last part of the week's perfusion. It should be noted, however, that these changes do not interfere with the life of the cells; positive epithelial cultures can be obtained from thyroids perfused for 18 or 21 days. Possibly the violent osmotic changes and the coagulation due to fixation are little to cause more considerable disturbances in the cytoplasm exposed for several days to artificial and not entirely adequate nutritional conditions than is the case during the usual process of fixation in those taken immediately from the animal. The blood vessels do not show sure signs of alterations. But the mesenchyme may show signs of proliferation, the degree and type of which is, however, highly variable.

Cultivation of the thyroid gland for several weeks is possible when the gland is transferred every week to another pump with a fresh supply of perfusion fluid. After 1 month, the thyroid tissue may

retain the histological features of a normal gland. The epithelium seems to assume an aspect of inactivity, the cells are flattened, and the colloid may be very dense and abundant (Fig. 4). The number of such experiments of long duration is, however, too small to enable us to put forward any complete statement of the morphology of this particular group.

Because of the considerable variability of the structure of cat and rabbit thyroid glands, both lobes should always be matched in experiments with culture of the isolated gland; one side to be used for the experiment, the other as a control. The fact that the process of perfusion is liable to bring about certain structural changes in the thyroid epithelium makes such an arrangement even more important.

Effects of Stimulation on Isolated Thyroid Glands

The process of cultivation, and especially changes in the composition of the perfusion fluid (notably the admixture of certain substances to the medium, as for example Witte's peptone), has the effect of inducing to a more or less marked degree an increase and probably a stimulation of the secretory elements. Biochemically, certain changes in the secretory activity can be detected. The results of the iodine analysis of different glands and the corresponding perfusion fluids will be published later.

As a method of stimulating the thyroid cells under well known experimental conditions, we have utilized the thyro-stimulating factor from the anterior pituitary. We have used the preparation of Collip, commercially prepared by Ayerst, McKenna and Harrison. The preparation was first tested on guinea pigs and we satisfied ourselves that a dose of 1 cc., injected subcutaneously, was able to release a very marked histological and cytological reaction in the thyroid gland of these animals. A great number of cat and rabbit thyroid glands were then cultivated under various experimental conditions in order to observe the effect of the pituitary stimulation upon isolated thyroid glands. Either the thyro-stimulating factor was added to the medium at the beginning of the experiment, or the gland was cultivated for 24 hours and then the dose of thyro-stimulating factor was added. Or else pituitary extract was added at once and then, after 24 hours, another dose was added to the medium. The amounts utilized have

been 1 to 2 cc. The duration of the experiments has been from 3 hours to 6 days. While one lobe has been cultivated for the experiment, the other lobe has always been perfused in another pump as a control.

Generally, the response of the thyroid epithelium to the stimulation has been similar, if not identical, to that observed in the living animal. In the preparations from glands cultivated for 24 hours, in which the thyro-stimulating factor has been added at the beginning of the experiment, there is a considerable vacuolization of the colloid, its stainability is often diminished and the size of the vesicles seems to be
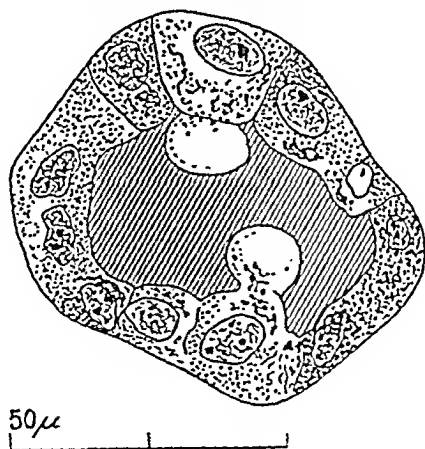
Text-Fig. 2. Vesicle from rabbit thyroid gland, perfused for 24 hours with 40 per cent serum in glucose-Tyrode solution plus 0.8 per cent anterior pituitary extract, showing vacuolization of colloid, swelling of epithelial cells and increase of the Golgi apparatus. Osmic impregnation; Heidenhain's iron-hematoxylin stain.

slightly decreased. There is, as a rule, a considerable swelling of the supranuclear regions of most of the thyroid cells (Fig. 5), and mitotic activity may be observed (Figs. 1 and 2). The mitochondria are increased in size and definitely filamentous. The Golgi apparatus is increased in size and is often of a distinctly reticular shape. Vacuoles appear both in the Golgi zone and in the basal regions of the epithelial cells (Text-fig. 2).

There are, however, differences between the morphological aspect of pituitary thyro-stimulation during perfusion and the corresponding reaction in the living animal. First, the reaction in the isolated gland

is never so pronounced as in the gland *in situ*; the degree of the cellular response and the uniformity of the tissue affected is inferior in the case of the cultivated glands. Next, there is a difference in time. In the living animal the thyro-stimulation with the pituitary factor takes place very quickly, so that the tremendous cytoplasmatic swelling which is characteristic of the initial stage of the reabsorptive phase of thyroid activity, occurs inside the 1st hour after stimulation; and during the following hour the intense vacuolization and release of the colloid material is evident. In the culture experiments, the cellular reactions may be comparatively slight, even 3 or 4 hours after the introduction of the thyro-stimulating factor. 6 to 8 or 10 hours later, the cellular stimulation is fairly well established. The state of morphologically increased secretory activity is, as a rule, plainly evident during the next 1 or 2 days. Even in experiments in which the thyroid gland has been cultivated for 6 days, there may be a considerable difference between the stimulated and the control gland. But the reaction does at that time show a tendency to diminish. Whether this is due to disappearance of the thyro-stimulating factor; whether it is a sign of exhaustion on the part of the thyroid cells; or whether the slowing down of the reaction is caused by an accumulation, in the medium, of active iodinized compounds, which in turn may react upon the thyroid epithelium and thus cause a differential mechanism, antagonizing the action of the pituitary factor, cannot be determined at present. Specially designed experiments are needed to elucidate this particular problem. But there is a definite response to the thyro-stimulating pituitary factor on the part of the thyroid epithelium, and the reaction seems to be fundamentally the same as that observed in the living animal.

CONCLUSIONS

1. During the 1st and 2nd day of cultivation of the whole thyroid gland in the Lindbergh apparatus, perfused by a medium containing 20 or 40 per cent homologous serum diluted with glucose-Tyrode, the state of the thyroid epithelium remains histologically normal and even the most delicate cytological details retain their normal aspect.

2. Cultivation over a period up to 6 days need not cause any serious histological alteration of the gland; but cytological examination

reveals an increasingly damaged condition of the mitochondria and the Golgi apparatus. These changes seem not to interfere with the life of the cells, however; epithelial culture from the perfused organs yields positive results.

3. The process of cultivation is liable to cause a slight morphological reaction in the thyroid epithelium, which resembles a feeble stimulation of the tissue.

4. Addition of the thyro-stimulating factor from the anterior pituitary to the perfusion fluid causes a stimulation of the cultivated thyroid epithelium which is comparable to, but not so marked as, the corresponding reaction in the living animal.

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EXPLANATION OF PLATES

PLATE 4

FIG. 1. Left thyroid gland from rabbit (Experiment 428 L), perfused for 48 hours with 40 per cent serum in glucose-Tyrode solution. Plenty of colloid; practically no vacuolization; epithelial cells cuboidal. Fixation: Zenker-formol solution; Heidenhain's iron-hematoxylin stain.   × 700.

FIG. 2. Right thyroid gland from same rabbit as in Fig. 1; perfused for 48 hours with 40 per cent serum in glucose-Tyrode solution plus 0.8 per cent thyro-stimulating factor. Vacuolization of colloid; hypertrophic epithelium (note mitotic figure); mitochondria very distinct. Fixation: Zenker-formol solution; Heidenhain's iron-hematoxylin stain.   × 700.
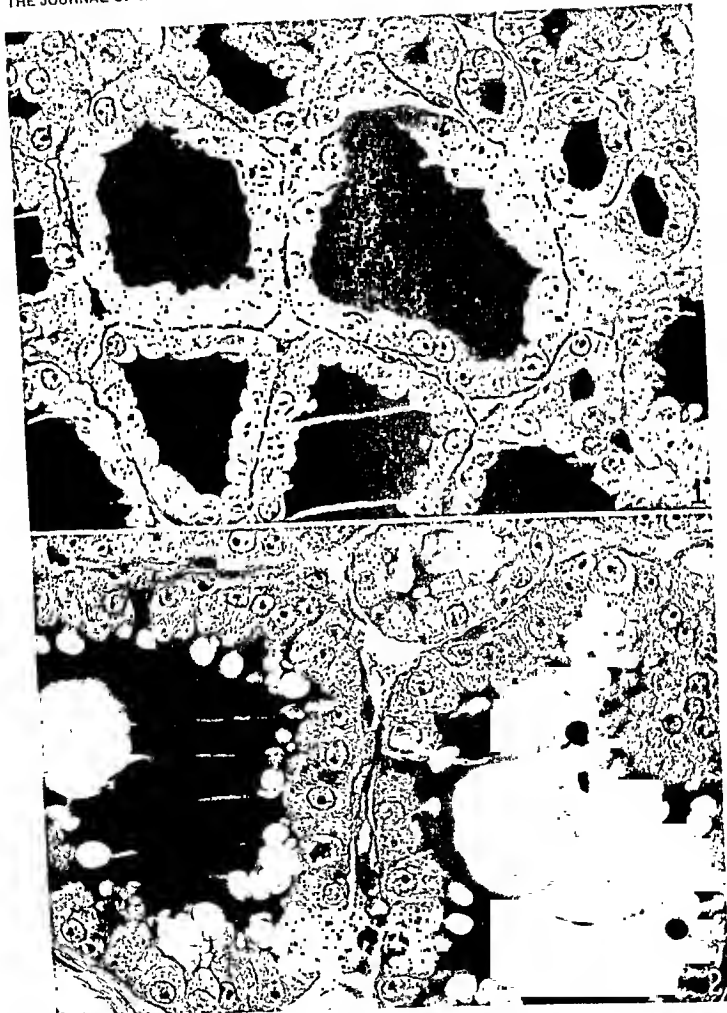
PLATE 5

FIG. 3. Normal shape and size of Golgi apparatus in thyroid cells from a rabbit's gland cultivated for 24 hours in 40 per cent serum plus glucose-Tyrode solution. Osmic saturation (Ludford's modification). × 900.

FIG. 4. Right thyroid gland from cat (Experiment 367 L), perfused for 1 month with 40 per cent serum plus glucose-Tyrode solution. Plenty of colloid; not much vacuolization: flattened epithelial cells. The structure of the left gland, which was not cultivated, was that of a very active thyroid parenchyma, with small vesicles, highly vacuolized colloid and low columnar epithelium. Fixation: Zenker-formol solution; hematoxylin-eosin stain. × 240.

FIG. 5. Vesicles from rabbit thyroid gland cultivated for 24 hours with 0.8 per cent thyro-stimulating factor added to the perfusion fluid. Slight reaction of epithelium; swelling and vacuolization of the supranuclear cytoplasm in a few cells. Fixation: Mann-Kopsch solution; Heidenhain's iron-hematoxylin stain. × 900.

FIG. 6. Vesicles from rabbit thyroid gland cultivated for 24 hours with 0.8 per cent thyro-stimulating factor added to the perfusion fluid. Considerable reaction with many swollen epithelial cells: the mitochondria are distinctly filamentous (note the white arrow). Technique and magnification as in Fig. 5.

THE CULTURE OF WHOLE ORGANS

III. The Problem of Antihormones Studied on Isolated Living Thyroid Glands

By HARALD OKKELS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

Seven years ago a considerable impetus was given to research on the biology of the thyroid gland by the use of extracts from the anterior pituitary. Soon, however, confusion arose on account of the peculiar refractoriness which animals treated with thyro-stimulating pituitary extracts for long periods are liable to develop. Today the study of this refractory state has become a major issue in thyroid biology, as well as in endocrinology generally.

Several investigators have shown that the initial hyperplasia of the thyroid gland produced in animals by the administration of the thyro-stimulating factor from the anterior pituitary may regress and involute to the colloid atrophic state, in spite of continued treatment. Similarly, the metabolic rate, after an initial rise to a maximum on the 7th to the 14th day, falls to normal or below. The mechanism whereby this regression in thyroid activity is brought about is unknown and the published observations and various explanations forwarded by different workers disagree to a very large extent. Some authors have suggested that the refractoriness may be the result of an exhaustion of the thyroid parenchyma. Others assume that specific neutralizing substances are formed and circulate in the blood of the refractory animals.

Collip and Anderson (1) have advanced the so called antihormone theory. According to their view, there may be an antagonistic principle for each hormone which is not the result of an antigen-antibody response but is a true hormone in every way. Collip (2) has lately declared that possibly the antihormone theory should be applied only to the trophic (pituitary) principles. He admits that theoretically a resistant state may be due to a variety of causes, but he stresses the point that the condition is transferable, *i.e.* through serum injections into test animals. Blood serum of guinea pigs which have become resistant to the effects of anterior pituitary extract neutralizes these extracts when injected into normal

guinea pigs.   Leo Loeb (3), Werner (4) and others were unable, however, to find inhibitory properties in the serum, using the technique of Collip.   Werner found that different pituitary preparations, all of them potent thyro-stimulators, have a different ability to induce a refractory state.   Katzman, Wade and Doisy (5) were unable to develop a refractory condition in rats by implanting homologous fresh pituitary glands during 9 months.   This observation is in keeping with experiments carried out by Krogh and Okkels.   Crude pituitary extracts are less liable to cause a state of refractoriness in guinea pigs than are highly purified preparations; further, in long duration experiments an administration of thyroxin to refractory animals is followed at any time by the characteristic rise in metabolism.

Collip and his collaborators claim that refractoriness is bound to develop if only the treatment with the thyro-stimulating factor is continued long enough; and whenever a refractory state has been reached the condition is claimed to be transferable.   Scowen and Spence (6) have been able to confirm Collip's observations; at the same time these authors found no evidence of exhaustion of the thyroid cells.

Parkes and Rowlands (7) and others have reported experiments which seem to fit in with the antihormone theory.   They found that an antigonadotropic serum, procured by injecting gonadotropic pituitary factor into rabbits over a period of 2 months, will completely inhibit the effect of the specific action of the anterior pituitary extract on the gonads; such serum may be said to effect passive immunization against the secretion of the animal's own pituitary.

The site of formation of the inhibitory principles is unknown.   Neither the thyroid nor the hypophysis seems to form inhibitory substances which pass over into the blood.   At one time the adrenal cortex was named as a possible source of antihormone during refractoriness.   Recently, Gordon, Kleinberg and Charipper (8) have found evidence which points to the spleen as a source of the antigonadotropic principle, results which they interpret as a manifestation of reticulo-endothelial activity.

To our mind, the problems posed by the antihormone theory—especially with regard to thyroid activity—are to a large extent focussed in the two questions:

1. Does a "cytological immunity" exist during the state of refractoriness such that the thyroid epithelium itself is unable to respond to any further stimulation by pituitary extracts?

2. Is the serum from refractory animals able to render normal thyroid cells immune to stimulation by anterior pituitary extracts?

In order to answer these questions, we designed the following plan of experiments on isolated thyroid glands, cultivated in the Lindbergh apparatus (9, 10).

Technical

The thyroid glands of refractory rabbits were cultivated in Lindbergh pumps: One gland was perfused in a pump with normal rabbit serum alone (positive control); the other gland was perfused in a pump with normal serum to which had been added thyro-stimulating pituitary factor. The serum from the refractory animals was saved and used in the following way: The thyroid glands of normal rabbits were cultivated in Lindbergh pumps. One gland was perfused in a pump with normal rabbit serum to which thyro-stimulating factor had been added positive control); the other gland was perfused in a pump with antiserum to which
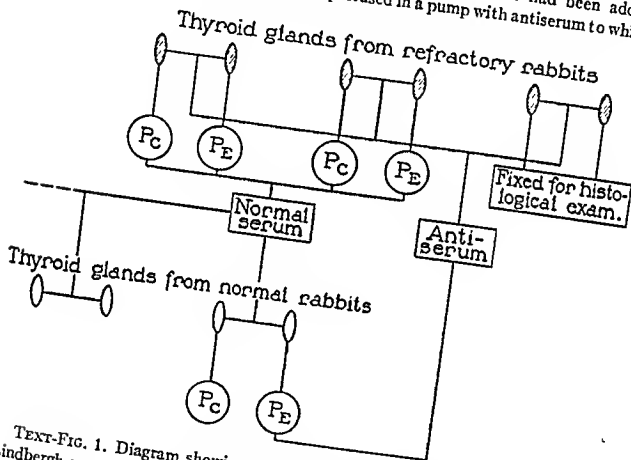
TEXT-FIG. 1. Diagram showing a single set of experiments. P_E and P_C are Lindbergh pumps with the cultivated experimental and control glands, respectively.

had been added the same amount of thyro-stimulating factor as in the control experiment. The plan of a single set of experiments is shown diagrammatically in Text-fig. 1.

Development of Refractoriness.—Rabbits weighing 2300 to 2700 gm. were used for treatment. It is true that rabbits are much inferior to guinea pigs as far as histophysiological work on the thyroid goes; but the use of guinea pigs was not possible for technical reasons. The high variability of the normal rabbit thyroid, which may be a serious source of error in ordinary experiments (Krogh and Okkels (11)), is, however, sufficiently controlled in the present series because the two

thyroid lobes of each animal were always matched as experimental and control specimens.

All the rabbits were maintained on a non-goitrogenic diet. 27 were treated with the thyro-stimulating pituitary factor. The extract was prepared by Ayerst, McKenna and Harrison, according to Collip's directions and standardized to a potency of 50 units per cc. Before the injections were started, we did, however, test different samples of the extract and satisfied ourselves that 1 cc. will bring about a very marked morphological reaction after 1 day in the thyroid of the guinea pig.

Each rabbit was injected with 3 cc. subcutaneously every 2nd day during the 1st week of treatment; then 3 cc. every 3rd day, until 30 cc. had been given. Then the dose was reduced to 1.5 cc. every 4th day during the remaining preparatory period (about 2 months), at the end of which time each animal had received 45 to 50 cc. of the pituitary extract. The weight of the rabbits showed a sharp decline during the first 2 weeks of the treatment; in 22 of the cases the weight increased during the next 2 or 3 weeks until a level was reached which was definitely higher than the initial one (Text-fig. 2); during the last weeks of the experiments the weight of the animals remained practically constant. The histology of the thyroid glands of the 5 animals which failed to develop resistance to the pituitary extract showed the usual hyperplastic state of the parenchyma. The histology of the thyroid glands (unperfused, negative controls; animals bled) from the larger group showed, with the exception of 1 case, a considerable increase in colloid and an atrophic state of the epithelium (Fig. 1). The rate of metabolism was not measured, but we believe that the weight charts, together with the histological findings, can be explained only by assuming that the 22 rabbits had developed refractoriness to the thyro-stimulating factor.

Cultivation.—Several batches of rabbits were prepared for the experiments. As soon as the treatment of one group had been completed, the animals were operated upon. Some of the treated rabbits were etherized and the thyroids dissected for perfusion.[1] Others were etherized and bled to death, the blood being saved in the freezing box overnight to prepare the special culture medium for the normal rabbit thyroid glands (see Text-fig. 1). In all cases the perfusion fluid consisted of 40 per cent rabbit serum diluted with glucose-Tyrode solution (with 5 mg. per cent phenol red as indicator). As a rule the thyroid glands were cultivated only over a period of 24 hours, preliminary study having shown that no sure signs of cytological damage occur during the first 24 to 48 hours of perfusion (Fig. 4). At the same time 24 hours are amply sufficient to permit the morphological effect of the thyro-stimulation to develop.

Histological Methods.—When the circulation was discontinued, the pumps were opened and the glands fixed in Zenker-formol solution and in Ludford's corrosive

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[1] All the animals were operated upon by Dr. Carrel. For details concerning the technique of dissection and the working of the apparatus, the reader is referred to Lindbergh (9) and Carrel (10).

chloride of mercury-osmic acid, with subsequent osmic saturation. Embedding in paraffin. Serial sections at 4 to 5 microns. Staining in hematoxylin-cosin and Heidenhain's iron-hematoxylin.

TEXT-FIG. 2. Chart showing the weights of the last batch of rabbits during 7 weeks of treatment with thyro-stimulating factor. One animal became sick; it had to be killed (K) and consequently was not utilized for any experiment.

Everyone working on the histophysiology of the thyroid gland knows that different parts of the parenchyma may present a different morphological aspect under varying experimental conditions. Usually the central parts of the gland seem to be more active than the periphery. This may be true for untreated animals; it is still more obvious in cases where a certain degree of stimulated condi-

tion is induced experimentally.   Therefore, it is imperative that the microscopical examination of thyroid glands, such as the ones with which we are concerned in the present series, is carried out with due consideration to this obvious source of error.   In our own case, the following estimates as to the degree of secretory activity in the different thyroid glands are based upon observations of topographically corresponding areas of the tissue in matched pairs of experiments.   The preparations have been compared again and again, and in many cases several observers have independently examined the same slides.   In cases in which degenerated areas of the perfused glands were of considerable extent, new series of sections were made to secure a correct morphological estimate of the functional state of the tissue.

RESULTS

The characteristic histological feature of the thyroid gland during the refractory state is a distention of the vesicles, an accumulation of very dense colloid, and a considerable flattening of the epithelium (Text-fig. 3).   Just as the normal thyroid tissue will react to the process of perfusion (12) by developing a slight swelling of the epithelium of the central parts of the gland, so is the thyroid of a refractory animal liable to undergo a slight change during cultivation in the Lindbergh apparatus.   There again, it is chiefly the central parts of the gland which are affected; furthermore, the change is only a slight one, causing the thyroid cells to assume a more cuboidal shape. Vacuolization of the colloid is exceptional.   If, however, thyro-stimulating pituitary extract is added to the medium, a considerable morphological reaction is observed in the epithelium of the refractory thyroid gland.   In several cases this reaction has been comparable to that taking place in normal glands during hormonal stimulation (Text-fig. 4).   Only once (Experiment 499) was this effect very slight (Figs. 5 and 6).   As a rule the isolated thyroid glands from refractory rabbits responded to the stimulating effect of anterior pituitary extract by a definite swelling of epithelial cells over large areas of the parenchyma, by enlargement of the Golgi apparatus and by an extensive vacuolization of the colloid (Figs. 7 and 8).

In the experiments in which the possible neutralizing effect of the serum from refractory animals was tested on isolated normal thyroid glands, the observations were less consistent.   In 3 of the experiments, we found practically the same degree of hypertrophy and increased cellular activity in both lobes of the thyroid (Figs. 11 and 12).   In 1
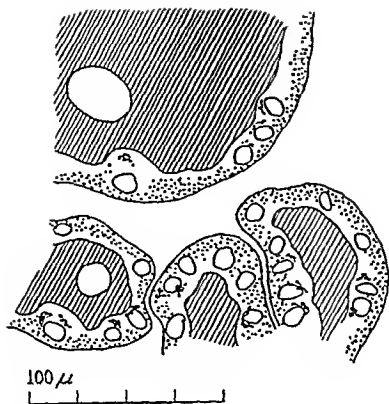
TEXT-FIG. 3. In spite of the fact that the thyroid parenchyma is atrophic during the condition of refractoriness, the Golgi apparatus is quite distinct and easily impregnable with osmic acid.

TEXT-FIG. 4. When the thyroid epithelium of glands from refractory rabbits responds to pituitary stimulation, the swelling of the cells and the increase of the Golgi apparatus may be considerable (compare with Text-fig. 3).

TABLE I

Response of Perfused Rabbit Thyroid Glands to Stimulation by Anterior Pituitary Extract

No.	Negative controls	Positive controls	Experiments	Amount anterior pituitary extract	Remarks
			Thyroid Glands from Refractory Rabbits		
				cc.	
486		0			
487			+	2	
488		+			
489			++	2	
496		(+)			Perfused for 22 hrs.
497	0				Not cultivated
498		0			Epithelium very atrophic
499			+ or 0	2	
525	+				Not cultivated
526			++	4	2 cc. anterior pituitary extract for 22 hrs. + 2 cc. for 5 hrs. Total: 4 cc. for 27 hrs.
531		+			
532			++	2	
533		0			
534			+	2	
540		+			
541			++	2	
542		0			
543			++	2	

0 indicates a morphologically inactive tissue of the colloid type.

+ to +++ indicate varying degrees of morphological activity.

The concentration of serum was 40 per cent in all cases. The amount of perfusion fluid 250 cc.

Where nothing special is indicated in the last column of the table the duration of the experiment was 24 hours.

TABLE I—*Concluded*

No.	Negative controls	Positive controls	Experiments	Amount anterior pituitary extract	Remarks
				cc.	
490		+++		2	Considerable degeneration
491	·		+	2	
494		+++		2	Considerable degeneration
495			+++	2	
500		++		2	
501			+	2	
527		+++		1	1 cc. anterior pituitary extract for 3 hrs.
528			++	1	1 cc. anterior pituitary extract for 24 hrs.
535		++		2	
536			++	2	
546		+++		2	
547			+++	2	

<div align="center">Normal Thyroid Glands Perfused with Serum from Refractory Rabbits</div>

case (No. 528) the morphology of the lobe perfused by antiserum showed a less active state than the corresponding control. In the remaining 2 experiments of the group (Nos. 491 and 501), the controls were definitely more active morphologically than the glands perfused with antiserum; probably, therefore, an inhibitory effect had been achieved here (Figs. 9 and 10).

All the positive controls in this experimental group showed distinct histological signs of considerable secretory activity, as would be expected in cases in which normal thyroid glands perfused with normal serum were exposed to the stimulating effect of a potent extract from the anterior pituitary.

The degree of the morphological reactions observed in all the perfused glands is indicated in Table I, where both groups of experiments and the results are recorded.

DISCUSSION

The histological examination of perfused and stimulated thyroid glands from refractory rabbits shows that the thyroid epithelium itself is not unable to respond to the stimulation by pituitary extract during the state of refractoriness in the animal.   We must, therefore, answer in the negative the first question put in the introduction of this paper: There is no evidence for the existence of a cytological immunity of the thyroid epithelium in rabbits which have been rendered immune to the action of the thyro-stimulating factor from the hypophysis. The thyroid epithelium may be very atrophic—exhausted—during the state of refractoriness.   But the thyroid cells of the isolated gland can be made to respond to pituitary stimulation within 24 hours when cultivated.

The second question, whether the serum from refractory rabbits is able to antagonize the effect of the thyro-stimulating factor from the hypophysis, is only partly answered by our results.   In 3 out of 6 cases, no sure signs of any antagonism between the antiserum and the pituitary extract were observed.   It would seem as if the antiserum in the remaining 3 cases had inhibited the action of the thyro-stimulating factor upon normal thyroid cells.   But the serum did not neutralize the effect of the pituitary extract completely.   And the degree of inhibition was surprisingly small, considering the large amount of serum utilized in each experiment.

In view of the fact that the thyroid cells themselves, as the first group of experiments show, are not fundamentally changed by the state of refractoriness as concerns their ability to react morphologically upon pituitary thyro-stimulation, it seems logical to assume either that the inhibitory effect of the antiserum is a simple quantitative matter of neutralization, or that an extra-thyroid mechanism is involved.   The first assumption is hardly tenable.   If we suppose that inhibition is determined simply by the amounts of antagonistic principles—the antihormone and the pituitary extract—actually present as a mixture in the perfusion fluid, it is hard to explain why similar experiments in living animals imply admixture of only a very much smaller amount of antiserum to obtain a complete neutralization.   It should be remembered that in each of our experiments at

least 80 cc. of serum from refractory animals were made to react upon one single lobe of thyroid gland in an attempt to counteract the effect of 1 or 2 cc. of an anterior pituitary extract. Scowen and Spence (13) have been able to prevent the occurrence of thyroid hyperplasia in guinea pigs by injecting subcutaneously 2 cc. of antiserum against 1 cc. of an active anterior pituitary extract given intraperitoneally. Similar results were obtained in rabbits. These investigators believe that the neutralizing substance is a hormone and not an antibody formed against a foreign protein.

All the same, in the experiments on the isolated thyroid gland, and with the use of much greater amounts of serum, it is possible to obtain only a comparatively small inhibitory effect, or none at all. Probably, therefore, organs or tissues other than the thyroid epithelium are involved in the mechanism underlying the phenomenon of refractoriness to the thyro-stimulating factor. It should be noted in this connection that the isolated, perfused thyroid glands cannot always be considered completely deprived of their nerve supply from sympathetic ganglia; and nerve cells have been observed to remain histologically normal during perfusion in the Lindbergh apparatus for a considerable length of time. Very little is known concerning the interrelationship between the nervous system and the thyroid, but living nerve tissue may actually be present in some of the perfused thyroid preparations. Whether this is of any significance as far as our particular problem goes, it is impossible to decide at present.

SUMMARY

1. Perfusion of isolated thyroid glands from rabbits, made refractory to the action of the thyro-stimulating factor from the hypophysis by repeated injections for 2 months, has shown that in this condition the thyroid epithelium responds to pituitary stimulation.

2. The serum from refractory rabbits does not neutralize completely the thyro-stimulating effect of anterior pituitary extract upon an isolated normal thyroid gland.

3. It is believed that tissues other than the thyroid play a rôle in bringing about the phenomena which have been described as refractoriness against a prolonged action of the thyro-stimulating pituitary factor.

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EXPLANATION OF PLATES

PLATE 6

FIG. 1. Uncultivated thyroid gland from refractory rabbit. Abundant colloid; practically no vacuolization; very atrophic epithelium. Fixation: Zenker-formol solution. Heidenhain's iron hematoxylin stain. × 650.

FIG. 2. Histology of normal rabbit thyroid gland (Experiment 428 L; colloid type) for comparison with Fig. 1. Same technique and magnification.

FIG. 3. Right lobe of uncultivated thyroid gland from rabbit (Experiment 497), showing atrophy of the epithelium during refractoriness. Same technique and magnification as Fig. 1.

FIG. 4. Left lobe of the thyroid gland from the same rabbit as in Fig. 3 (Experiment 496 L), cultivated for 22 hours with 40 per cent serum plus glucose-Tyrode solution. The size of the epithelial cells is somewhat increased but there is no vacuolization. Same technique and magnification as Fig. 1.

FIG. 5. Experiment 498 L. Atrophic thyroid gland from a refractory rabbit. Duration of cultivation, 24 hours. The cells are flattened and the colloid is not vacuolated. Fixation: Zenker-formol solution; hematoxylin-eosin. × 195.

FIG. 6. Experiment 499 L. Other lobe of thyroid gland from same animal as in Fig. 5. Duration of cultivation: 24 hours; 2 cc. of thyro-stimulating factor was added to the perfusion fluid. Example of very slight stimulation (compare with Table I). The general histological aspect of the gland is the same as in the control, but there is a definite increase of the fine vacuoles in the colloid. Histological technique and magnification as Fig. 5.
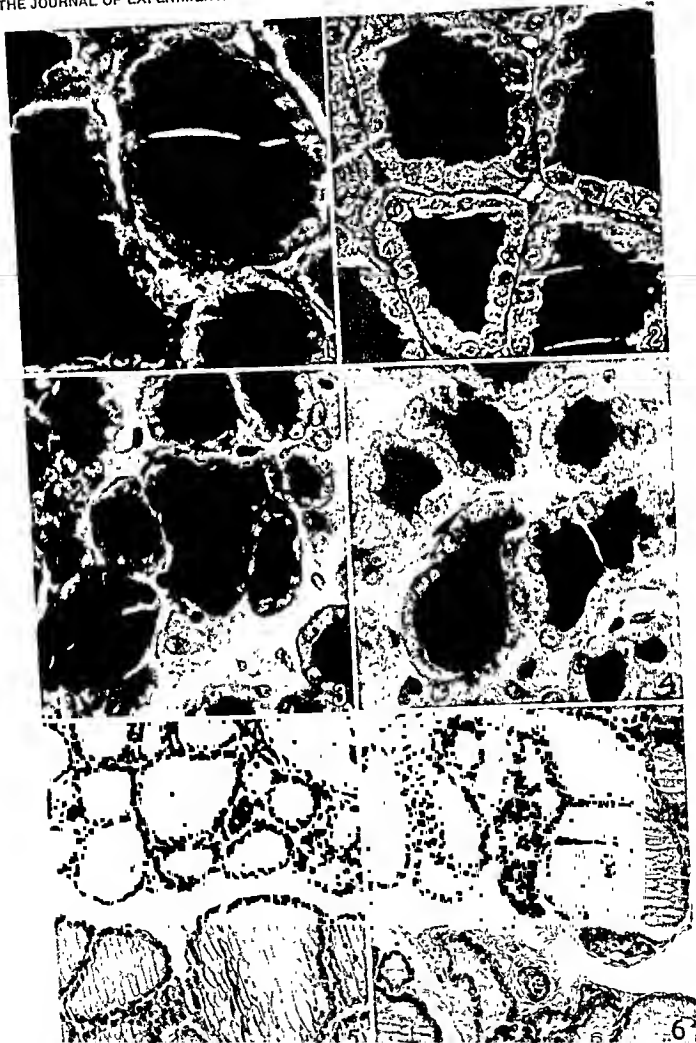
PLATE 8

FIG. 7. Experiment 542 L. Histologically inactive thyroid gland from refractory rabbit. Same technique and magnification as Fig. 5.

FIG. 8. Experiment 543 L. Other lobe of thyroid gland from same animal as in Fig. 7. Medium degree of response to stimulation by anterior pituitary extract. Same technique and magnification as Fig. 5.

FIG. 9. Experiment 490 L. Hyperplastic thyroid gland from normal rabbit. Typical effect of thyro-stimulating pituitary factor. Same technique and magnification as Fig. 5.

FIG. 10. Experiment 491 L (perfused with antiserum). Other lobe of thyroid gland from same animal as in Fig. 9. The effect of the thyro-stimulating factor is very considerably inhibited. Example of maximum effect of the antiserum; compare with Table I. Same technique and magnification as Fig. 5.

FIG. 11. Experiment 546 L. Hyperplastic thyroid gland from normal rabbit. Typical effect of thyro-stimulating pituitary factor. Same technique and magnification as Fig. 5.

FIG. 12. Experiment 547 L (perfused with antiserum). Other lobe of thyroid gland from same animal as in Fig. 11. The tissue shows about the same degree of hyperplasia and stimulation. Same technique and magnification as Fig. 5.

PLATE 7

IMMUNIZATION OF GUINEA PIGS WITH A MODIFIED STRAIN OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS

By ERICH TRAUB, V.M.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

Two strains of lymphocytic choriomeningitis virus obtained in 1935 from naturally infected stock mice have been used for experimental work in this laboratory. These strains were highly virulent for guinea pigs when isolated. The present paper deals with the effect on the virus of serial passage through guinea pigs and mice and the use of a modified strain as an immunizing agent for guinea pigs.

The Strains Employed

Strain A, which was used in most of the experiments reported in previous papers (1, 2), showed no marked change in the course of 8 serial passages through guinea pigs by subcutaneous injection[1] or 10 serial transfers in virus-free mice by intracerebral inoculation. The mouse passages were discontinued after the 10th transfer, but the guinea pig passage strain has been maintained.

Strain B was obtained from two guinea pigs, both of which developed severe choriomeningitis after intracerebral inoculation with the brain of a mouse and were killed when moribund on the 12th or 13th day. Virus from their brains has thus far been passed in series through 14 guinea pigs by subcutaneous injection and in another series through 34 virus-free mice by intracerebral inoculation. Brain suspensions were used for subinoculation in guinea pigs and mice.

Effect on Strain B of Serial Passage through Guinea Pigs

The transfers through guinea pigs have maintained the high virulence of strain B for this species. Subcutaneous or intracerebral injections are usually fatal, even when very small amounts of virus are given, such as 1 to 10 minimal infective doses (M.I.D.), and the guinea

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[1] Subcutaneous inoculations were made into the metatarsal pads.

pigs that do not die develop a very severe disease with marked emaciation and slow recovery.

All mice injected intracerebrally with this strain become sick, but many of them survive and fail to show the striking convulsions which characterize the disease induced by intracerebral injection with the mouse passage strain.   Intravenous or intraperitoneal injection causes illness in the majority of the mice, but the rate of mortality is considerably lower than after intracerebral inoculation.   Subcutaneous injection and intranasal instillation produce no symptoms and yet render mice resistant to intracerebral inoculation.   The virus can be demonstrated in the blood of infected mice and occasionally persists in it for more than a month after recovery.

Effect on Strain B of Serial Passage through Mice

The results of intracerebral tests show that mouse passage has increased the virulence of strain B for mice.   Subclinical infections occasionally occurred in the early passages even when large amounts of virus were inoculated, but injections with more than 10 M.I.D. of virus from the later passages caused typical convulsions and death on the 6th or 7th day in nearly 100 per cent of the mice.   Doses near the borderline of infectivity, however, occasionally produced immunity without apparent symptoms.   Intravenous, intraperitoneal, subcutaneous, or intranasal inoculations of virus from the later passages have failed to cause any definite signs of illness, but have immunized against intracerebral injection with virus.   The blood of three mice injected intracerebrally was infectious when symptoms were manifest, but the virus was not demonstrated in the blood in repeated tests made 1 month after inoculation.

The pathogenicity of the mouse passage strain for guinea pigs has greatly decreased.   It is not known exactly when this loss of virulence occurred; brain suspension from the 8th serial transfer was avirulent for guinea pigs.   Intracerebral injections of this modified virus are followed by fever lasting several days and no other sign of disease. Some guinea pigs do not gain weight during the febrile period as rapidly as uninjected controls, but to an uninformed observer they do not appear ill.   None of the symptoms that characterize the infection with the original virus or the guinea pig passage strain, such

as labored respiration, drowsiness, salivation, vomiting, conjunctivitis, and emaciation, have been noted, and all of the 53 guinea pigs injected either intracerebrally or subcutaneously survived. Subcutaneous inoculations cause fever in the majority of the cases, but it is usually lower and of shorter duration than that induced by intracerebral injection. The amount of virus inoculated either intracerebrally or subcutaneously did not markedly influence the reaction of the animals. The blood contained virus during the febrile reaction on the 7th day after injection in two guinea pigs tested.

The virulence of the modified strain for guinea pigs was not restored by a single guinea pig passage, no matter whether blood or brain was used for subinoculation. Intracerebral or subcutaneous injection with a mixture of mouse brain containing modified virus and guinea pig brain in which the virulent strain had been inactivated by heating at 70°C. for ½ hour, likewise failed to increase the virulence of the modified strain. Nor has it been possible to modify the virulent guinea pig passage strain by one or two intracerebral passages through mice. This fact indicates that the modification of the mouse strain probably did not take place during the first two serial transfers in mice.

In the following section of the paper the strain B passed through mice and attenuated for guinea pigs will be called "mouse virus," while the strains A and B passed through guinea pigs and highly virulent for this species will be referred to as "guinea pig virus." For immunity tests with guinea pigs, strains A and B were often mixed to insure a high degree of virulence in the inoculum.

Immunization of Guinea Pigs with Mouse Virus

A single intracerebral or subcutaneous injection with mouse virus was sufficient to induce a high degree of resistance to intracerebral or subcutaneous inoculation with a large amount of guinea pig virus, such as 0.1 or 0.5 cc. of a 10 per cent guinea pig brain suspension, given 3 weeks later. The development of fever in some guinea pigs following subcutaneous inoculation apparently had no relation to the degree of immunity produced.

The immunity arose rapidly as Table I shows. Subcutaneous injection of mouse virus into the left hind pad followed immediately by

a similar injection of guinea pig virus into the right hind pad failed to prevent the disease induced by the latter strain, but there is evi-

TABLE I

Immunity of Guinea Pigs in Relation to Circulating Antivirus

Guinea pig No.	Route of inoculation with mouse virus	Test for antivirus in serum drawn 2–6 hrs. before test of immunity	Test of immunity by injection with guinea pig virus				
			Time after inoculation with mouse virus	Route of inoculation	Reaction	Controls	
						No.	Reaction
1					D 11	17	D 14
2					D 13	18	D 15
3	sc		1 min.	sc	D 27	19	D 9
4					Mild disease, rapid recovery	20	D 11
5					Severe disease, slow recovery	21	-D 14
6		—			Fever	22	D 12
7	sc	—	4 days	sc	Mild disease, rapid recovery	23	D 12
8		—			Slight fever	24–27	D 11–15
9	sc	—	8 days	ic	None		
10		—			None	28–30	Very severe disease, slow recovery
11		—			Slight fever		
12	sc	+			Slight fever	31	D 11
13		—			None		
			10 days	ic			
14		+			None		
15	ic	—			None	32	D 12
16		—			None		

sc = subcutaneously.
ic = intracerebrally.
D 11 = died on 11th day.

— = no antivirus detected.
+ = antivirus probably present.

dence that its course was modified in two animals (Nos. 4 and 5). On the 4th day a degree of resistance was apparent, and on the 8th or 10th day, 6 of the 9 guinea pigs tested were completely resistant,

while the remainder developed a transient fever. Other guinea pigs not recorded in the table, which were tested for immunity by intracerebral inoculation on the 18th, 21st, or 31st day after subcutaneous or intracerebral injection with mouse virus, were all completely resistant.

The Relation of Circulating Antivirus to the Immunity of Guinea Pigs.—The failure to detect circulating antivirus in mice possessing a high degree of immunity to choriomeningitis was reported in a previous communication (1). Numerous other sera from mice immunized or hyperimmunized by a variety of methods have since been tested but none of them had definite neutralizing power. If antivirus was present, its concentration was too low to account for the high degree of resistance shown by the mice. Extracts of the brains and livers of mice that had become resistant to intracerebral injection by subcutaneous inoculation with mouse virus likewise failed to give any evidence of an antiviral substance. The sera of immune guinea pigs, on the other hand, usually contained antivirus when tested a few weeks after recovery.

Because of this apparent difference in mechanism between the immunity of mice and that of guinea pigs, an attempt was made to determine whether the immunity of the latter was due solely to circulating antivirus or, as in mice, to another factor as yet unknown but closely associated with the tissues affected by the virus.

A number of guinea pigs injected subcutaneously or intracerebrally with mouse virus were bled by heart puncture under deep ether anesthesia a few hours before they were tested for immunity as indicated in Table I. The sera obtained were avirulent for mice, except that of guinea pig 11 which produced the disease in one of the two mice inoculated. Neutralization tests were performed with the sera according to the method already described (1).

The results of the tests recorded in Table I indicate that the high degree of immunity present on the 8th and the 10th day after injection was not associated with demonstrable antivirus in the majority of the animals. Other guinea pig sera drawn 1 month after injection with mouse virus had definite neutralizing properties.

DISCUSSION

Strains of choriomeningitis virus isolated in earlier experiments from mice of the infected stock (2) often differed in virulence for guinea

pigs although similar in every other respect. Some caused merely a febrile reaction in the majority of the animals, while others induced a very severe, fatal disease. However, not one of the strains of low virulence was quite consistent in its effect, a fatal disease sometimes resulting with those that were relatively non-pathogenic. The variations in severity of the disease were thought to be mainly due to differences in the susceptibility of guinea pigs, and this assumption was supported by the fact that the virus obtained from the same mouse at different times was differently pathogenic for guinea pigs (1, Table III). However, the rapid and striking change of strain B brought about by a few serial passages through mice now suggests that variations of the virus may be in part responsible for those differences. It has been shown that guinea pigs from the same stock as those used before react uniformly both to the guinea pig passage strain, which invariably causes very severe choriomeningitis, fatal in 80 to 90 per cent of the cases, and to the modified mouse strain, which has failed to produce symptoms in any of the 53 guinea pigs injected.

The modified virus is a very good immunizing agent for guinea pigs, since it is harmless to the animals and produces a solid immunity after a single injection.

The immunity to choriomeningitis of guinea pigs and mice differs in that immune guinea pigs as a rule develop circulating antivirus, whereas the sera from immune mice thus far tested have had no definite neutralizing properties. The comparatively late appearance of the antivirus in the guinea pig indicates, however, that it does not solely account for their immunity, which seems to be closely associated with the tissues affected by the virus. Similar observations have been reported for other viruses, for example, foot-and-mouth disease (3) and poliomyelitis (4–6). The possibility of infecting immune animal tissues *in vitro* with a variety of viruses, *e.g.*, virus III (7), vaccinia (8), herpes simplex (9), the salivary gland virus of guinea pigs (10), and pseudorabies (11), does not necessarily weigh against this conception of the mechanism of antiviral immunity. The washing and soaking of the tissue fragments, which usually preceded the infection in such experiments, may have removed an antiviral substance that was fixed in the tissues or rendered it ineffec-

tive by dilution. The evidence thus far obtained with tissue extracts from immune mice does not favor the assumption that such a substance is present, but further tests are necessary to disprove its existence. It is also possible that the removal of the tissue from its physiological environment and its maintenance under extremely unfavorable conditions alter the permeability to viruses of the cellular membrane. This permeability may provide the basis of susceptibility and immunity.

SUMMARY

A strain of choriomeningitis virus which was highly virulent for guinea pigs, as isolated from a naturally infected white mouse, has been markedly attenuated for guinea pigs by serial intracerebral passage through white mice. The change of virulence occurred before the 8th serial passage. The modified virus as a rule produces fever in guinea pigs but no other symptoms, and the infection is followed by a very solid immunity. Parallel passages of the same strain through guinea pigs have maintained its high virulence for this species but slightly reduced its pathogenicity for mice. These observations indicate that the differences in virulence for guinea pigs noted before (1, 2) with different strains of choriomeningitis virus obtained from infected stock mice may be due not only to differences in the susceptibility of guinea pigs but also to variations in the virulence of the virus.

A marked degree of resistance was demonstrable in several guinea pigs on the 4th, 8th, and 10th day after injection with modified virus, when antivirus could not yet be detected in the serum. Circulating antivirus appears therefore to play a secondary part in their immunity, which seems to be closely associated with the tissues as in mice.

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ELEMENTARY BODIES OF VACCINIA FROM INFECTED CHORIO-ALLANTOIC MEMBRANES OF DEVELOPING CHICK EMBRYOS

By JOSEPH E. SMADEL, M.D., AND M. J. WALL

(From the Hospital of The Rockefeller Institute for Medical Research)

PLATE 8

Paschen bodies (1) or elementary bodies, have been observed in many types of cells in various animal species infected with vaccine virus (2). Suspensions of these minute structures in a relatively pure state have been prepared by means of differential centrifugation from infected skins of rabbits (3–5) and guinea pigs (6). The presence of large amounts of protein in emulsified organs has made it difficult in the past to obtain from such sources clean suspensions of virus elementary bodies. Infected skin, however, is peculiarly well adapted to the methods previously employed since the highly infectious dermal pulp, which results from scraping the skin (4, 5), contains less tissue proteins than do ground infected organs.

The preparation of suspensions of vaccine virus elementary bodies in a relatively pure state from infected tissues of a generic class different from that previously used would add to the existing evidence for the intimate association of the elementary body with the causal agent. Moreover, such an experiment would offer an opportunity for further observation on the soluble precipitable substances (6) that accompany the virus infection. Studies (7, 8) on the chick chorio-allantoic membrane inoculated with vaccine virus indicated that this tissue, which has a high infective titer and contains numerous elementary bodies, might be suitable material from which to obtain suspensions of relatively pure Paschen bodies. The purpose of this paper is to describe a method for preparing suspensions of elementary bodies from vaccine virus-infected chorio-allantoic membranes of developing chick embryos, and to present the results of serological studies on the elementary bodies and virus-free filtrates of infected membranes.

Materials and Methods

Chorio-Allantoic Membranes.—The chorio-allantoic membranes of 12 to 14 day chick embryos were exposed by Burnet's modification (9) of the technic of Woodruff and Goodpasture (10). Usually 0.1 cc. of a 10^{-3} dilution of virus suspension was dropped on the membrane surface; then the egg was gently rotated to insure spread of the inoculum. After incubation for 36–48 hours at 37°C., the infected portion of the chorio-allantoic membrane was removed. Occasional membranes that were contaminated with bacteria were discarded.

Virus.—Vaccine virus of the C. L. strain,[1] which had been carried for 4 years in this laboratory by passage of elementary bodies on rabbit skins, was rendered bacteriologically sterile by storage with ether. This virus served initially to infect chick membranes. Experiments were performed with the 2nd to the 27th membrane subculture of virus.

Antisera.—Antivaccinal sera were obtained from rabbits and guinea pigs after hyperimmunization with washed elementary bodies of rabbit origin. Monkeys recovered from intravenous inoculation of similar preparations of virus also yielded immune sera. Antisera against the heat-labile and heat-stable precipitable substances, respectively, of vaccinia which had been prepared in rabbits (11) were supplied by Dr. Robert F. Parker.

Infective Titers.—Infective titers were determined by intradermal injection into rabbits of serial tenfold dilutions of virus. Duplicate titrations were always performed.

Preparation of Elementary Body Suspensions

Suspensions of elementary bodies of vaccinia in a relatively pure state were prepared from infected chorio-allantoic membranes of chicks in the following manner.

The infected portions of 10–25 membranes were placed in Locke's solution immediately following removal from the eggs. After the last membrane was harvested the entire lot was rinsed in fresh Locke's solution in order to remove additional mucilaginous material adherent to the surface as well as blood which had oozed from the cut vessels of the membranes. The tissues were next individually dipped into ethyl ether to remove part of the lipid substances and were again rinsed in Locke's solution. Excess fluid having been drained from the membranes, they were placed in a mortar, minced with scissors, and ground vigorously. 10–20 cc. of dilute buffer solution, pH 7.2, (standard citric acid-disodium phosphate buffer, pH 7.2, diluted 50 times with distilled water) were added to the triturated material and the suspension was thoroughly shaken in a test tube. Sediment thrown down at 1000 R.P.M. in the International horizontal centrifuge was resus-

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[1] Virus was obtained from Dr. J. Craigie in 1933.

pended in a similar volume of buffer solution and recentrifuged. Supernatant fluids from these two centrifugations were pooled and again spun at 1000 R.P.M. for 10 minutes. The resultant supernatant suspension was distributed in flat pyrex tubes, that have an inside diameter of 4 mm. and a capacity of 5 cc., and run in a Swedish angle centrifuge for 1 hour at 3500 R.P.M. Fluid was poured off and saved for titration of precipitinogens. The angle sediment, which contained practically all of the elementary bodies as well as considerable amounts of tissue materials, was taken up in 5–10 cc. of dilute buffer solution, pH 8.0. 0.5 cc. of a filtered 1 per cent solution of commercial trypsin that had been freed from lipids by successive extraction with ethyl ether and petrol ether was added to the suspension; the mixture was incubated at 37°C. for n half to 1 hour. Fat-extracted trypsin was used because Pirie (12) found that the lipids in commercial trypsin— not the enzymes—inactivated vaccine virus. The digested material was then washed 3 to 7 times by sedimentation in the angle centrifuge and resuspension in dilute pH 7.2 buffer solution. After the final washing, the suspension was spun in the horizontal centrifuge for half an hour at a speed of 2500 R.P.M. in order to remove particles larger than elementary bodies.

In the manner described, suspensions of relatively pure elementary bodies were prepared by a method of differential centrifugation similar to that used by other workers (4, 5). However, one important additional procedure, namely, tryptic digestion, was employed. Suspensions of virus still contained some soluble tissue proteins after 3 washings. This was indicated by the presence of amorphous, brown, precipitate interspersed among the round, black, elementary bodies in smears stained by Morosow's technic (13). When such preparations were subjected to further washing the amorphous precipitate was reduced to a minimum. Fig. 1 is a photograph of a stained preparation made from a suspension of elementary bodies treated with trypsin and washed 7 times in all.

Protein digestion was an important step in purification since repeated washing of the sediment from the angle centrifugation with dilute buffer solution alone failed to remove adequately tissue proteins. Physiological salt solution was not employed because Craigie (14) found that vaccine virus elementary bodies in the presence of 0.85 per cent saline readily underwent spontaneous agglutination. Treatment with trypsin usually reduced the amount of the angle sediment to one-half to two-thirds of its original volume.

Only a portion of the elementary bodies present in infected membranes was secured by the method described as routine. In 3 in-

stances, cells and debris, sedimented during preliminary horizontal centrifugations and discarded in the regular procedure, were first subjected to tryptic digestion and then to differential centrifugation. The resultant material, after repeated washing and additional enzymatic treatment, was found to be rich in elementary bodies. The infective titer, moreover, was in each case the same as that of the elementary body suspension prepared according to routine. These suspensions were not so clean as the regularly prepared ones, as evidenced by the precipitated amorphous material in stained smears. Such suspensions were not satisfactory for use in immunological tests because marked spontaneous agglutination occurred.

Infective Titer of Elementary Body Preparations

The most important single fact that has led to an acceptance of the close relationship between elementary bodies of vaccinia and the agent responsible for the disease is the correlation of the infective titer of suspensions with their elementary body content. The virus accompanies the elementary bodies from rabbit dermal pulp (3–5) even when filtered (15); the bodies increase in tissue culture with multiplication of the virus (16); and a definite numerical relationship exists between the bodies and a single infectious dose (17); moreover, materials freed from elementary bodies are not infectious (4, 5).

Additional evidence for the idea that elementary bodies represent the virus itself or are closely associated with the virus was brought out in the present set of experiments. The infectivity of membrane material was not appreciably altered by tryptic digestion and repeated washing; the final preparation of elementary bodies was as infectious as the original membrane emulsion. Experiment 5, summarized in Table I, is illustrative of the data supporting this statement. After the removal of coarse particles by preliminary centrifugation, the membrane emulsion had an infective titer of 10^{-6}. The material sedimented in the angle centrifuge was digested with trypsin and washed 5 times; this represents a dilution of the original angle sediment of the order of 1×10^{-4}. At this stage the infective titer was 10^{-7}. Further washing diluted the first angle sediment to approximately 1×10^{-6} times its original volume; nevertheless, the titer remained 10^{-7}. Two factors operated to increase the titer of the original emul-

TABLE I

Infectivity of Suspension of Elementary Bodies of Vaccinia

Experiment 5	Day	Treatment	Volume of suspension (cc.)	Dilution of 1st angle sediment	Infectivity of suspension							
					10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
	1	Membrane emulsion after horizontal centrifugation	22	0	+++	++++	++++	++++	++++	++++	±	
21 membranes infected with 12th passage virus	2	Washed total of 5 times. Treated with trypsin after 2nd sedimentation	5	1×10^{-4}	++++	++++	++++	++++	++++	++++	+	
		Stored at 3°C.	"	"								
	4	Washed 2 additional times (total 7)	"	1×10^{-6}	++++	++++	++++	++++	++++	++++	+	
	11	Stored at 3°C.	"	"	++++	++++	++++	++++	++++	++++	+	⧺

TABLE II

Agglutination of Elementary Bodies of Vaccinia

Suspension	Antivaccinal serum	Dilution of serum										Type of agglutination
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	
Chick E.B. No. 9	Rabbit 6022	—	±	+++	++++	++++	++	++	++	+	—	Granular
Rabbit E.B. No. 7660	"	+++	+++	+++	++++	++++	++++	++++	++	+		Large flocks
Chick E.B. No. 8	Guinea pig 5972	—	+++	+	++	+	±					Granular
Chick E.B. No. 9	"			+	++		—	—	—			"
Rabbit E.B. No. 7078	"	+++	++++	++++	+	+	+	—	—			Large flocks

E. B. = elementary bodies of vaccinia.

sion. Tryptic digestion probably liberated virus from cell particles and from cells killed by immersion of the membranes in ether, for, as observed in another experiment, tryptic digestion alone raised the titer of a crude membrane emulsion from 10^{-5} to 10^{-6}. The second factor was the decrease in volume of suspending fluid; the particulate matter originally present in 22 cc. was later taken up in only 5 cc. of fluid.

Decrease in the number of elementary bodies and in the infective titer occurred to a certain extent during the manipulation incident to purification of elementary body suspensions. This was apparent when relatively few infected membranes constituted the source of virus, e.g., in Experiment 3, in which 8 membranes were employed. The titer following tryptic digestion, and before washing, was 10^{-6}; after 4 washings, which diluted the original angle sediment approximately 1×10^{-7} times, the titer had fallen to 10^{-5}. Another source of loss was the thin layer of elementary bodies that always adhered to the walls of the glass tubes. Moreover, the small fraction of elementary bodies which had been sedimented along the outer wall of the centrifuge tube, but which had not yet been thrown to the bottom, was often lost in pouring off the supernatant fluid. Finally, the last horizontal centrifugation also contributed to the losses, because in several instances, the sediment which resulted from this procedure was found to be rich in elementary bodies, when stained by Morosow's technic, and had an infectivity equal or almost equal to that of the suspension prepared according to routine. Nevertheless, the final horizontal centrifugation was useful, notwithstanding the waste that accompanied it, because debris and clumped elementary bodies were removed.

Agglutination of Elementary Bodies

Agglutination of elementary bodies (18, 3) by antivaccinal serum was another significant step in establishing the importance of these structures in the disease. It seemed worth while to determine whether or not elementary bodies prepared from hosts of different generic classes were equally agglutinable with a given immune serum.

Agglutination Reaction.—Agglutination of elementary bodies was carried out in the manner described by Craigie (4) and Parker and Rivers (5). Dilutions of

immune sera were made with freshly prepared physiological salt solution buffered to pH 7.2 by the addition of 1 cc. of standard citric acid-disodium phosphate buffer solution to each 100 cc. of saline solution. Elementary body suspensions were brought to the proper concentration for testing by the addition of dilute buffer solution, pH 7.2. 0.25 cc. of elementary body suspension was added to 0.25 cc. of diluted serum which had been placed in a pyrex tube with outside dimensions of 10 x 75 mm. Tubes were placed in a covered rack and incubated overnight at 50°C.

The data presented in Table II show that elementary bodies prepared from either chick membrane or rabbit skin are agglutinated approximately to the same extent by immune sera from two mammalian species. Hyperimmune rabbit serum, in a dilution of 1:1280, agglutinated elementary body suspension 9 obtained from membranes of chick embryos; the serum had the same titer when tested with an elementary body suspension derived from dermal vaccine pulp of rabbits. The guinea pig and monkey antivaccinal sera, although not so potent as the rabbit immune sera, respectively agglutinated chick membrane and rabbit dermal elementary bodies to an approximately equal degree. A consistent difference between the two virus suspensions was noted in the type of clumping. Elementary bodies of rabbit origin formed large loose flakes when mixed with immune sera from various species; in higher concentrations of serum the floccules settled and left a clear supernatant fluid. Chick membrane elementary bodies, however, agglutinated only in a granular form. Repeated washing tended to reduce the agglutinability of bodies; the highest titers were obtained when suspensions were washed 3 or 4 times.

Soluble Precipitable Substances in Filtrates of Infected Membranes

It has been pointed out by Sabin (19) that soluble antigens obtained from tissues infected with vaccinia might be "specific products of infection which are not derived from the virus substance" rather than products of the virus. Craigie and Wishart (6) subsequently demonstrated the immunological identity of L and S precipitinogens found in dermal pulp filtrates from infected rabbits, guinea pigs, and calves, and Ch'en (20) found a soluble antigen in tissue culture vaccine virus prepared according to the method of Li and Rivers (21) in which minced chick embryo tissue suspended in Tyrode's solution was used.

Further information regarding these soluble antigens was sought by means of precipitin tests on the virus-free filtrates from infected chick membranes.

Precipitin Reactions.—Precipitin tests were carried out on extracts of infected membranes. The supernatant fluid which resulted from the first angle centrifugation was filtered through a Seitz pad; the filter had been previously prepared by the passage of 10 cc. of broth and 2 cc. of normal rabbit serum. These filtrates were not infectious. Serial dilutions of the clear serum-colored filtrates were prepared with buffered saline solution and mixed with equal volumes of diluted immune serum, the optimal dilution of the immune serum having been determined by preliminary titration. Tubes and racks similar to those employed for agglutination tests were used; the mixtures were incubated overnight at 50°C.

Precipitin tests on Seitz filtrates of suspensions of infected membranes demonstrated the presence of heat-labile and heat-stable antigens. Results of such a test are shown in Table III. Hyperimmune rabbit serum, which contained antibodies against L and S fractions, gave a slightly higher titer with pooled rabbit dermal filtrate than with membrane filtrate. Rabbit serum that had L but no S antibody reacted equally well with chick membrane and rabbit skin filtrates. Pure anti-S rabbit serum, like the L-S serum, precipitated membrane filtrate at a slightly lower titer than rabbit filtrate.

The results of precipitin tests, which were done on all filtrates of infected membranes, were found to vary. No attempt was made to remove inhibitive substances (6) from the membrane filtrates; this may account for considerable variation in titer of different preparations. Membrane emulsions were filtered with difficulty through Seitz pads, even after removal of most of the particles by centrifugation. The absence of demonstrable precipitinogens in Experiments 6 and 9 probably depended on technical manipulation, such as extraction with ethyl ether and the use of several pads for filtering a small volume of material. There was no obvious explanation for the inability to demonstrate the presence of precipitinogens in filtrate 10; of interest also was the failure to find agglutinogens in the elementary body suspension prepared from this group of membranes which had been inoculated with the 24th membrane passage virus. This failure to demonstrate serologically active substances was not dependent on a change in the virus, because precipitinogens and agglutinogens were

TABLE III

Soluble Precipitable Substances in Filtrates of Chick Membranes Injected with Vaccine Virus

| Filtrate | | Type of antivaccinal rabbit serum | Dilution of filtrate | | | | | | | | |
Source	No.		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Chick membrane	8	L-S serum 6022, diluted 1:12		++++	++++	++++	++	+	−	−	−
Rabbit skin	Pool I	" " " " "		++++	++++	++++	++++	++	++	+	−
Chick membrane	8	L serum 5110, diluted 1:2	++	++	++	+	++	−			
Rabbit skin	Pool I	" " " " "	++	++	+++	++	++	−			
Chick membrane	8	S serum 5199, diluted 1:4	++++	++++	+++	++	−	+	−		
Rabbit skin	Pool I	" " " " "	+++++	++++	++++	++++	++	+	−		

present in lot 11 which had been infected with the 26th membrane passage.

DISCUSSION

The present observations suggest that by use of appropriate methods the infectivity of tissues from animals with vaccinia may be found, in each case, to be associated with their elementary body content. The experiments described in this paper throw no light on the idea of Craigie and Wishart (22) that there are two forms, resistant and active, of vaccine virus elementary bodies. Nor do the observations bring direct evidence to bear on Sabin's suggestion (19) regarding host production of the soluble precipitable substances. The finding of L and S precipitinogens in the filtrates of infected chick membranes by means of immune sera prepared in rabbits by repeated injection of elementary bodies from rabbits, might be considered additional circumstantial evidence that these antigens are products of the virus rather than the result of a response of the host to infection with the active agent.

SUMMARY

By a method of differential centrifugation and tryptic digestion suspensions of elementary bodies have been prepared from chorioallantoic membranes of chick embryos infected with vaccine virus. The infective titer of the final suspension of elementary bodies was usually the same as that of the original tissue emulsion. Elementary bodies from infected chick membranes were agglutinated as well by antivaccinal serum obtained from different mammalian species as were bodies prepared from inoculated rabbit skin. Seitz filtrates of infected chick material contained soluble precipitable substances of vaccinia; these filtrates and filtrates from infected rabbit skin, respectively, reacted equally well with rabbit serum which contained either L or S antibodies.

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EXPLANATION OF PLATE 8

FIG. 1. Photograph of a preparation of elementary bodies in a relatively pure state obtained from chorio-allantoic membranes of chick embryos infected with vaccine virus.   Morosow stain.   × 1200.

1
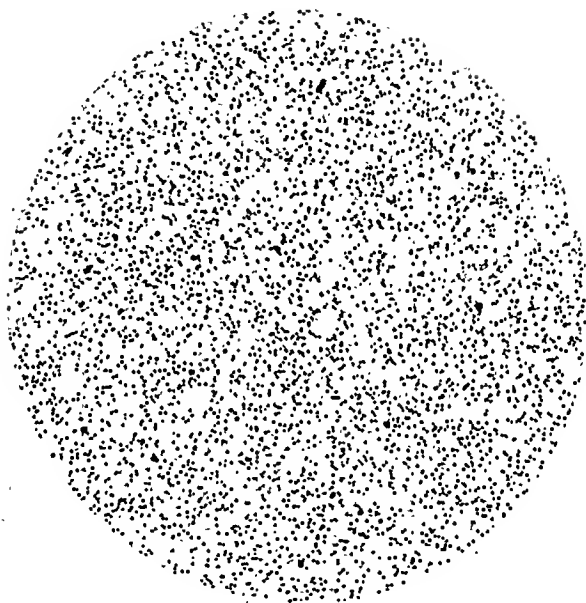
(Smadel and Wall: Elementary bodies of vaccinia)
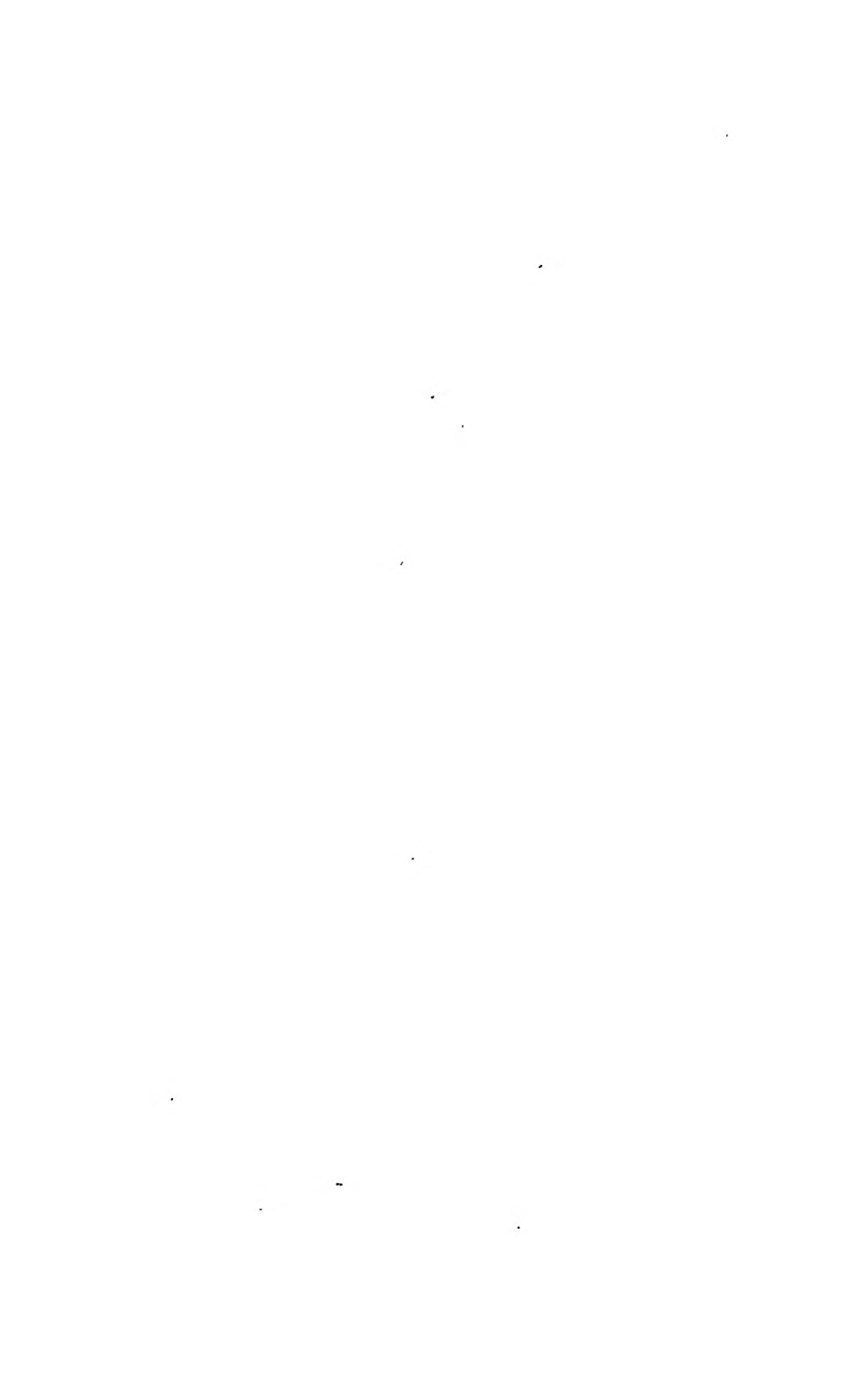
STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS

IV. ANAPHYLAXIS INDUCED BY PICRYL CHLORIDE AND 2:4 DINITROCHLOROBENZENE

By K. LANDSTEINER, M.D., AND M. W. CHASE, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

In previous communications (1–4), report has been made of sensitization effects in guinea pigs following intracutaneous or superficial administration of a number of simple chemical compounds, e.g., nitrosodimethylaniline and 2:4 dinitrochlorobenzene. Thus, animals treated intradermally with small quantities of the substances exhibit distinctly increased reactions to subsequent intradermal injections, and give erythematous reactions when a solution of the incitant is spread on the skin. A study of various nitrochlorobenzenes then demonstrated a parallelism between skin sensitizing capacity and chemical reactivity, consistent with the idea that sensitization effects are due to conjugated antigens formed *in vivo*. That artificially conjugated antigens—azoproteins—can sensitize (anaphylactically) to the conjugate, the reactions being specific for the substance linked to protein, had been shown previously (5; *cf.* 6), but there was no direct evidence that sensitivity to simple substances may depend upon the formation of such antigens (7). Another result was that in the case of acyl chlorides, which also are able to sensitize, skin sensitiveness and anaphylaxis were produced simultaneously upon repeated intracutaneous injections, indicating a relationship in the causation of the two effects. In the present paper, this question is further investigated.

EXPERIMENTAL

Anaphylaxis Experiments with Picryl Chloride and 2:4 Dinitrochlorobenzene.—In the experiments cited above, benzoyl chloride and

p-chlorobenzoyl chloride gave rise to both skin sensitization and anaphylaxis. The formation in these instances of conjugate antigens evidently responsible for the production of anaphylaxis is not particularly surprising, since the substances are easily decomposed by water and are highly reactive, *e.g.* with proteins. In this respect the experiments are somewhat similar to the sensitizations obtained with diazonium and diazoamino compounds (8, 9). It seemed necessary to make investigations on the possibility of producing anaphylaxis with substances that are relatively stable and are known to cause allergic phenomena in human subjects. Two substances were chosen for study, picryl chloride, previously shown to sensitize guinea pigs (1) and capable, moreover, of producing sensitization in human beings upon intracutaneous administration,[1] and 2:4 dinitrochlorobenzene, which is known to cause contact dermatitis in a large number of industrial workers handling it (10–12). Picryl chloride, although combining readily with proteins in alkaline solution, is not decomposed by water at room temperature for a considerable period of time, and can be recrystallized from boiling alcohol; it does, however, react slowly with serum proteins at serum alkalinity. The second substance, 2:4 dinitrochlorobenzene, is stable in boiling water for hours and does not combine with serum proteins to any appreciable extent without the addition of alkali.

Guinea pigs were sensitized by repeated intracutaneous administration on the dorsum, the course commonly consisting of about fifteen daily injections, six a week, each of 1/400 mg. picryl chloride in 0.1 cc. saline.[2] The commercial preparation was used after two recrystallizations from a benzene-alcohol mixture, m.p. 82° (uncorrected). For the injections, solutions of the requisite concentration were prepared by diluting in saline an alcoholic 0.3 per cent solution.

White male guinea pigs weighing 350–450 gm., mostly albinos, were used for the sensitizations. The development and the degree of skin sensitivity to picryl chloride correspond in a general way to observations reported with 2:4 dinitrochlorobenzene (1). With daily injections, the reactions being recorded 24 hours

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[1] Personal communication from Dr. Marion B. Sulzberger.

[2] Lately it has been found that very satisfactory skin sensitization can be attained by intracutaneous injection of larger quantities of picryl chloride than those used before, given simultaneously in several sites (1/50 mg. injected in each of 7 sites, test made 1 month later).

later, evidence of a heightened response would usually be noted between 6 and 8 days after the first injection, the sensitivity gradually developing to give elevated, pink areas 10 to 15 mm. in diameter, often with blanched or livid centers, at times with a necrotic spot. A period of 2 or 3 weeks was allowed between the last injection of the course and the final testing, which was made by spreading on the belly, after clipping the hair, 1 drop of a 2 per cent solution of picryl chloride in olive oil; for subsequent tests, new belly sites were chosen each time. The reactions were read 18 to 24 hours later, following use of a depilatory 2 or 3 hours before. On normal animals, similarly treated as controls, reactions were faint or absent. Of 134 pigs treated with picryl chloride by the above or a comparable method, only seven did not show evidence of sensitization; this uniformity closely resembles experiences with 2:4 dinitrochlorobenzene (4).

For the preparation of protein conjugates 1 millimol of picryl chloride in 5 cc. chloroform was shaken vigorously for 15 minutes with a mixture of 30 cc. horse serum (or guinea pig serum) and 15 cc. N/2 Na_2CO_3; the temperature being kept at about 5°C. After the removal of insoluble material by centrifugation in the cold, the supernatant was acidified to maximum precipitation, and the picryl protein sedimented by spinning. The protein compound was dissolved in water with addition of alkali to pH 8–9, the reaction adjusted close to neutrality, and a small amount of insoluble material centrifuged off. For purification, the protein was precipitated by treatment with 5 volumes of alcohol, centrifuged, and redissolved in water at slight alkalinity; it was finally precipitated with acid, washed once with saline by centrifugation, and dissolved in saline at slight alkalinity. The solution was made approximately neutral, becoming somewhat turbid thereby, and the concentration (about 2 to 3 per cent) was determined.

Recently we have found that the protein compounds can be made by gentle shaking of serum with finely ground picryl chloride; this method can also be used with 2:4 dinitrofluorobenzene (see below).

With intracutaneous injections of picryl chloride, as previously found with *p*-chlorobenzoyl chloride (2), guinea pigs were found to develop anaphylactic, in addition to skin, sensitivity. As seen in Table I, typical anaphylactic shock resulted when picryl protein conjugates were given intravenously. While with the batch of animals shown in the table 4 mg. represented approximately the limiting quantity of antigen to produce fatal shock, this value fluctuated with different series of sensitized animals. It was as low as 1/5 mg. in a group which had received six weekly intradermal injections of picryl chloride (tested 10 days later with "picryl horse serum"), and also in a lot given ten to fourteen daily injections (test made with "picryl guinea pig serum" after 4 weeks' rest); in other experiments,

only a small proportion of the animals were fatally shocked with 10 or 20 mg. doses of antigen.

As a complication, in some cases it seemed that large doses were less effective than smaller ones in demonstrating anaphylaxis (*cf.* 8). The interval between the last sensitizing injection and the intravenous injection of picryl protein may

TABLE I

Anaphylaxis in a group of guinea pigs given daily intradermal injections of 1/400 mg. picryl chloride for 15 days, and injected intravenously with picryl protein between 8 and 12 days after the last skin injection. Figures in parentheses indicate change in temperature (°C.).

No.	Amount injected	Intravenous injection of picryl horse serum
	mg.	
1	12	Slight symptoms (−1.1)
2	10	Typical anaphylaxis † 4 min.*
3	8	" " † 6 "
4	8	" " † 13 "
5	8	" " † 4 "
6	8	Severe shock, recovered (−5.9)
7	4	Typical anaphylaxis † 4 minutes
8	4	" " † 6 "
9	4	Slight symptoms (−1.2)
10	2	Coughs, eyes running, labored breathing (−1.5)
11	1	Typical anaphylaxis † 7 min.
12	1	Slight symptoms (−1.0)
13	1/5	No symptoms (+0.2)
		Controls
14	16	No symptoms (−0.6)
15	16	" " (−0.2)
16	8	" " (−0.4)
17	8	" " (−0.4)
18	4	" " (−0.6)

* The symbol † signifies death of animal; in all cases the autopsy findings have been characteristic.

be as short as a week; in one experiment in which animals were tested after intervals of 1 week and 5 weeks, the degree of anaphylactic sensitivity had diminished by the 5th week.

The presence of antibodies in the serum of guinea pigs sensitized by intradermal injections of picryl chloride was demonstrable by passive transfer, using the Schultz-Dale method (see 13).

The sensitized animals were bled between the 6th and the 14th day after the final intradermal injection of picryl chloride, and the serum, commonly in 3 cc. amounts, was injected intraperitoneally into virgin female guinea pigs weighing 180–250 gm. After 24 or 48 hours the animals were exsanguinated by heart puncture and the excised uterine horns were rinsed and mounted in a Dale apparatus in separate chambers of 20 cc. capacity, the recording being made at about threefold magnification. The oxygenated bath usually was the fluid recommended by Dale (13) (NaCl 0.9 per cent, KCl 0.042 per cent, $CnCl_2$ 0.012 per cent, $NaHCO_3$ 0.05 per cent, in glass-distilled water); infrequently the amount of calcium chloride was reduced to one-half. The antigen (1 or 2 mg., or less, of picryl protein made with horse serum) was added to the bath in a volume of 0.2 cc. or less, the resultant concentration being without effect upon the uterine horns of normal guinea pigs. If a contraction ensued, and specific desensitization was to be demonstrated, the horn was allowed to relax fully and was then washed by repeated changes of oxygenated fluid; thereupon the same dose of antigen which had caused the first contraction was added to the bath. Finally, to test the condition of the muscle, an addition of histamine was made.

A representative experiment of this sort is given in Text-fig. 1, where it is seen that the passively sensitized muscle responded to picryl protein even at 1:80,000 dilution and that when the test was repeated the uterus was seen to be desensitized. Anaphylactic antibodies were found with most batches in a large proportion of animals sensitized by intradermal injections of picryl chloride as described above, e.g., with one group, eight out of ten guinea pigs, and in another experiment seven out of nine, gave positive transfers; in some lots definitely poorer effects were obtained. On the whole, these results would seem to compare not unfavorably with those obtained in homologous passive transfer experiments with sera from animals injected with proteins (14, 15).

Many of the transfers effected with 3 cc. amounts of serum resulted in maximal contractions maintained 2 to 6 minutes when the horns were exposed to picryl horse serum in the usual concentration of 1:20,000. With the most active sera, 1 cc. was adequate for sensitization. In some instances where uteri appeared to be sensitized highly, the antigen was employed in greater dilutions; in one case, there was a maximal sustained contraction to an antigen concentration of 1:2,000,000, which indicates a degree of sensitivity close to that shown by highly sensitive uteri as a result of customary protein immunization (cf. 13). It may be mentioned that there

seemed to be some parallelism between the intensity of skin reactions elicited by the later preparatory injections and the content of anaphylactic antibodies in the serum; in some series of animals, however, such a relationship was not seen in the degrees of anaphylactic and cutaneous sensitivities, the latter tested by applying oil solutions on the skin several weeks after the last treatment.

Precipitins were detected in some of the sera which were among the most active in conferring passive sensitization, picryl protein being used as antigen in ring tests. The rings, although faint and detectable only with clear sera, were definite; the optimal concentration of picryl guinea pig serum was usually 1:30,000. Attempts to transfer skin sensitiveness passively by means of sera containing anaphylactic antibodies and precipitins have so far been negative.

While in the above experiments skin sensitization and anaphylactic antibodies were developed by intradermal injections as stated, it is of interest that in a preliminary experiment anaphylactic antibodies were found also in guinea pigs sensitized by continued daily application of one drop of a 2 per cent solution of picryl chloride in olive oil to the intact skin, anaphylactic sensitivity arising in a normal guinea pig from the intraperitoneal injection of 7.5 cc. pooled serum taken from three cutaneously sensitized guinea pigs.

Analogous experiments were carried out on guinea pigs sensitized to 2:4 dinitrochlorobenzene by means of repeated intracutaneous injections, as in the case of picryl chloride.

The development of skin sensitivity to this substance has been described (1); cutaneous sensitivity was tested by superficial application of a 1 per cent solution in olive oil. On account of its greater reactivity as compared with the chloro compound, 2:4 dinitrofluorobenzene (16) was used in the preparation of 2:4 dinitrophenyl protein conjugates. The latter were prepared with guinea pig or horse serum, the method for picryl protein being followed in the main. The guinea pig serum preparation was made by shaking in the cold for 10 minutes; in reprecipitating the protein derivative, careful adjustment of the pH and addition of salt were necessary; the antigen was used after dialysis against isotonic saline. The horse serum preparation was obtained in a similar manner. The solution tended to become turbid when kept in the ice box at neutral reaction, and before use the amount required was considerably clarified by cautious addition of NaOH and then adjusted with HCl until almost neutral.

In Schultz-Dale experiments with actively sensitized guinea pigs, since the animals usually were rather heavy by the time of testing, and the horns showed
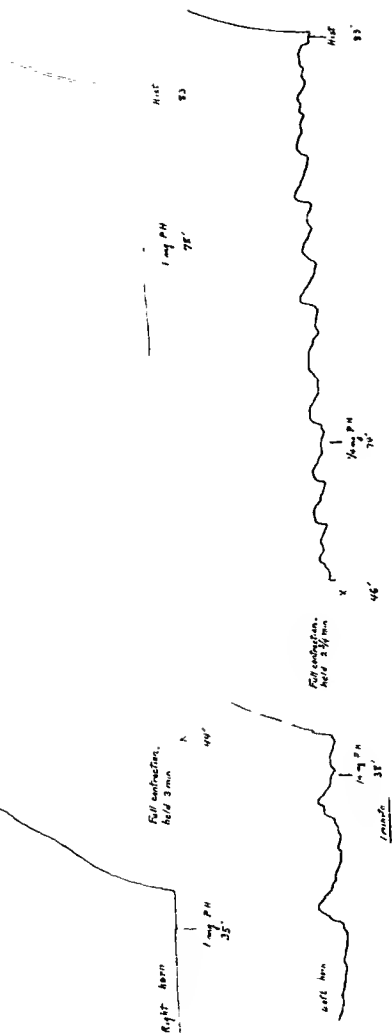
TEXT-FIG. 1. Anaphylactic antibodies in serum of guinea pig given fifteen daily intradermal injections of picryl chloride (see text), 7 days after the last injection, demonstrated by passive transfer. Recipient guinea pig (235 gm.) was injected intraperitoneally with 3 cc. serum, and the uterine horns were tested 2 days later. Additions of picryl horse serum (P.H.) to the bath (20 cc. Dale solution) were made at the times noted after mounting the horns; the sign X designates washing out of the chamber; histamine dihydrochloride (Hist.) was finally added (concentration 1:5,000,000 of the free base).

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...aphylactic response in a guinea pig sensitized by fifteen daily intradermal injecti...

...thod with 2:4 dinitrophenyl horse serum (D.N.H.) as antigen...

...ly weight) were mounted in 20 cc. modified Dal...

...e (Hist.) was used in a concentr...

spontaneous contractions in the above mentioned Dale solution, modified bath fluids were employed, either the Dale solution with the amount of calcium chloride reduced to one-half or one-quarter (animals weighing 325–400 gm.) or the calcium-free solution used by Bristol and Fleischner (17). For the latter (see 18), a stock solution is made with 10.5 gm. NaCl, 0.5 gm. KCl, 0.1 gm. $MgCl_2$, 5 cc. N H_3PO_4, 50 cc. water, and the bath fluid is prepared from 50 cc. stock solution, 5 cc. N Na_2CO_3, and 1000 cc. water. Along with repression of spontaneous contraction in these solutions, the specific reactions are apparently diminished and the tests less sensitive.

TABLE II

Anaphylactic response of a group of guinea pigs given 6 weekly intradermal injections of 1/400 mg. 2:4 dinitrochlorbenzene, 2:4 "dinitrophenyl guinea pig" serum being injected intravenously 4 weeks after the last skin injection.

No.	Amount injected	Intravenous injection of 2:4 dinitrophenyl protein
	mg.	
19	20	Severe symptoms (chronic type), recovered
20	20	Typical anaphylaxis, † 4 min.
21	20	No symptoms
22	20	Typical anaphylaxis, † 4 min.
23	10	Questionable symptoms
		Controls
24	20	No symptoms
25	20	" "

Guinea pigs sensitized by intracutaneous injections in the manner stated were tested after 7 to 40 days by intravenous injection with the protein conjugate. Resultant anaphylactic symptoms were variable, and the instances of fatal shock were few. Nevertheless, as seen in Table II (which presents the best result thus far obtained), the fact that the intracutaneous administration of 2:4 dinitrochlorobenzene can induce an anaphylactic state could be demonstrated beyond doubt. It would seem probable that continued study should determine experimental conditions under which the results will be more regular and passive transfer can be demonstrated.

Further evidence was forthcoming from experiments made with the Schultz-Dale method, the horns of sensitized guinea pigs being tested with 1:10,000 or 1:20,000 dilutions of 2:4 dinitrophenyl pro-

TABLE III

Anaphylactic desensitization by subcutaneous injection of picryl protein in two batches (A and B) of guinea pigs sensitized by 15 daily intradermal injections of 1/400 mg. picryl chloride. 12 days after the last skin injection, part of the animals were given 10 mg. picryl guinea pig serum subcutaneously; next day, all were tested for skin sensitiveness, and one day later picryl horse serum was injected intravenously. Figures in parentheses indicate change in temperature (°C.). The skin reactions on normal animals ranged from negative to faint pink.

	Animals given desensitizing injection				Sensitized controls		
No.	Application of a 2 per cent solution of picryl chloride in olive oil on the skin	Intravenous injection of picryl horse serum		No.	Application of a 2 per cent solution of picryl chloride in olive oil on the skin	Intravenous injection of picryl horse serum	
		Amount	Symptoms			Amount	Symptoms
		mg.				*mg.*	
Group A							
26	p., el.*	10	None**	30	dp., m.el.	10	† 4 min.
27	p., el.	10	None**	31	p., sl.swol.	10	† 11 min.
28	pp., sl.el.	10	None (+0.8)	32	pp.–p., el.	10	† 4 min.
29	pp.–p., sl.el.	5	None (−0.9)	33	p., el.	5	Moderate to severe (−0.7)
				34	pp., sl.el.	10	Moderate (−1.7)
				35	pp., sl.el.	10	Moderate (−1.9)
				36	pp.–p., sl.el.	10	† 30 min.
Group B							
37	p., sl.el.	10	None (−0.1)	40	pp., sl.el.	10	Slight to moderate (−1.8)
38	p., sl.el.	10	None (+0.5)	41	p., sl.el.	10	† 14 min.
39	p., el.	10	None (−1.0)	42	p., sl.el.	10	Moderate (−2.1)

*The following abbreviations are used: faint pink (fp.), pale pink (pp.), pink (p.), dark pink (dp.), slightly elevated (sl.el.), elevated (el.), markedly elevated (m.el.), swollen (swol.), blanched center (bl.c.).

**Temperature change not determined.

tein compounds prepared from horse or guinea pig serum. Here
again the results were inconstant, and negative with two small batches
of animals, which incidentally did not exhibit a high degree of skin
sensitivity; in other lots of guinea pigs, however, definitely positive
reactions occurred, *viz.* with two out of eight, and in five out of a
group of fifteen (tested 9 days after the last intradermal injection),
and in four out of nine (examined after a rest of 32 to 50 days). The
reactions ranged from relatively weak contractions to, in the majority

TABLE IV

Failure of subcutaneously administered picryl protein to desensitize the hyper-
sensitive skin of selected guinea pigs previously given intradermal injections of
picryl chloride. After the first skin test made by applying 1 drop of a 2 per cent
solution of picryl chloride in olive oil to the skin of the belly, half of the animals
were reserved for comparison, the others were injected subcutaneously with picryl
guinea pig serum (10 mg. on the 2nd, 4th, and 8th days, 20 mg. on the 10th day),
and a second skin test was made in the same way on the 12th day.

Animals given desensitizing injections			Sensitized controls		
No.	First skin test	Second skin test (after four injections of picryl protein)	No.	First skin test	Second skin test
43	p., bl.c., swol.	dp., m.el.	47	p., el.	pp.
44	pp., sl.el.	pp.	48	pp., sl.el.	pp.
45	pp.-p., sl.swol.*		49	pp.-p., sl.swol.	p., swol.
46	p.	p.	50	pp.	pp.-p.

* Animal died within a few hours after the first subcutaneous injection of picryl
protein.

of cases, maximal contractions sustained for 1 to 3 minutes, specific
desensitization being demonstrated regularly; such a record is shown
in Text-fig. 2.

Desensitization Experiments with Picryl Protein.—With animals
sensitized to picryl chloride, experiments aimed at desensitization
were carried out by administering picryl guinea pig serum subcu-
taneously prior to the intravenous shocking injection. The sub-
cutaneous injections were not seldom followed by local reactions,
consisting of edema, more pronounced than in normal animals, and
sometimes reddening of the skin. The results as presented in Table
III show that anaphylactic desensitization could be achieved reg-

ularly.   In contrast to this, it will be seen that the reactivity of the skin to superficial application of picryl chloride was not concomitantly abolished, and indeed even repeated subcutaneous injections of picryl protein had no noticeable influence on the degree of skin sensitiveness (Table IV).   While we have not investigated the subject particularly, in an experiment with a few animals we were able to desensitize the skin by long continued daily applications of an olive oil solution of the incitant, 2:4 dinitrochlorobenzene.   In a similar way Kobayashi (19), working with guinea pigs sensitized with an extract of *Rhus vernicifera*, reported desensitization by long continued painting with the extracts.   By means of subcutaneous injections in human beings, Blank and Coca (20) found that a certain degree of immunity to contact with poison ivy develops.

<div align="center">COMMENT</div>

The above experiments demonstrate that certain simple substances which cause human hypersensitiveness, with skin manifestations, produce upon intracutaneous injection into guinea pigs both skin sensitivity and anaphylactic sensitization.   This has been shown with picryl chloride and with 2:4 dinitrochlorobenzene, which are capable of inducing cutaneous sensitization in human beings.   It is noteworthy, indeed, that in these cases very small quantities of simple compounds can produce anaphylactic sensitization, evidently through combination with some substance of the animal body.

From our results, it appears that although both the compounds mentioned sensitize guinea pig skin in like manner they probably differ quantitatively in their capacity to evoke an anaphylactic state. While this distinction is one of degree only, for also with 2:4 dinitrochlorobenzene unquestionable anaphylactic effects were obtained, nevertheless the result taken in conjunction with the skin effects would indicate differences in the mode of anaphylactic sensitization and sensitization of the skin to superficial application of the incitant.[3] There are several other facts, from the experiments with picryl chloride, pointing in this direction.   In the first place it has not been possible to induce skin sensitivity to the simple substance, although

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[3] *Cf.* Landsteiner and Levine (6), page 353; Landsteiner (4).

an anaphylactic state is set up in this way, by injecting the protein conjugate intradermally, in contrast to the outcome of the converse experiment (Table I). Again, several attempts at passively sensitizing the skin to contact with the simple substance, by means of sera containing anaphylactic antibodies, have failed. Then in some experiments we observed a lack of parallelism in the degrees of anaphylactic and skin contact sensitizations in animals prepared by intradermal injections of picryl chloride. There are, finally, the desensitization experiments with protein conjugates (Table III) which were successful so far as anaphylaxis is concerned but were without effect on the dermal reactions produced by superficial application of the incitant to the intact skin. It would be premature to elaborate hypotheses concerning the differences in the processes leading to the two sorts of sensitization; tentatively it might be considered that the cutaneous manifestations are due to antibodies, perhaps of a special sort, produced by and fixed in the skin (*cf.* 6), or one could possibly think of the formation of various sorts of antigenic conjugates having the same "hapten component." The answer to these and other possibilities must await further study. Yet it would be most unlikely that the two specific sensitization effects induced at the same time by intradermal injections of "non-antigens," namely skin sensitivity and general anaphylaxis, are without a fundamental relationship.

That the two conditions are related is strongly indicated by the fact that both were found to be induced by substances characterized by their ability to form conjugates (2). Antibodies, it is true, have so far been demonstrated in our experiments only for the anaphylactic sensitization which results from intracutaneous (or even superficial) treatment with suitable chemical substances. However, in the experimental allergic dermatitis of guinea pigs as in human contact dermatitis the instrumentality of antibodies in the broadest sense of the word, namely specific substances formed in consequence of previous contact,[4] must be assumed *a priori* because of the phenomenon of specificity, although they have not as yet been experimentally established. Considering other cases, such as the absence of cir-

[4] *Cf.* Doerr (21, 22); Zinsser (23).

culating antibodies in later stages of the anaphylactic state, it is obvious that failure to demonstrate passive transfer of skin manifestations by means of serum is no decisive proof against the existence of antibodies, confined to the skin or perhaps circulating in small amounts and only transiently. Thus from the foregoing one may conclude that in the cases examined in this and in previous studies (1–3) the immunizing activity of conjugated antigens comes into play, this concept affording a plausible explanation for the immunological effects of simple substances.

SUMMARY

It has been shown that by the cutaneous administration of simple chemical compounds in small quantities—2:4:6 trinitrochlorobenzene (picryl chloride) and 2:4 dinitrochlorobenzene, the latter a typical incitant of contact dermatitis in man—it is possible to induce true anaphylactic sensitization in guinea pigs, demonstrable by the intravenous injection of protein conjugates and by the Dale technique, using isolated uterine horns. This furnishes strong evidence for the formation of antigenic conjugates following application of substances of simple chemical constitution. Since the anaphylactic state is induced by the same method of administration that gives rise to cutaneous sensitivity, the assumption would appear justified, when one takes into account the chemical properties of the inciting substances, that the formation of conjugated antigens offers an explanation for the skin effects also.

In the experiments with picryl chloride, anaphylactic antibodies, and occasionally precipitins, have been demonstrated.

The differences between the cutaneous and anaphylactic types of sensitivity are discussed.

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SPREADING PROPERTIES OF LEECH EXTRACTS AND THE FORMATION OF LYMPH

By ALBERT CLAUDE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

Extracts of certain mammalian organs, notably the testicle, have the power to increase tissue permeability (1). The rapid fading out of the wheal of injection when such an extract has been injected contrasts with the persistence of the bleb which follows the introduction of a fluid not possessed of the spreading property. The way in which tissue permeability comes to be modified is not yet understood. The permeated skin shows minor histological changes, which are restricted to the corium, the epithelial components remaining unaffected. Injection of highly concentrated testicle extracts produces, in addition to the spread, a local edema (2). This fact suggests that the phenomenon of spread may be related in some way to the processes which control the formation and removal of lymph.

Heidenhain endeavored to prove that lymph was a product of secretion of the endothelium, and not the result of mere filtration through the vessel walls. He found that certain substances, for example, peptone, egg albumin, extracts of liver and intestine, but more especially, extracts of mussels, leeches, and crabs, would produce a lasting increase in the lymph flow though they had no effect on the blood pressure. He termed such substances lymphagogues of the first class (3). A second group of lymphagogues included substances which increased the lymph flow from the thoracic duct by raising the pressure of the blood. In view of the possible relationship between the spreading phenomenon and the formation of lymph, some of Heidenhain's lymphagogues have been tested. In a preliminary investigation it was noted that egg albumin failed to produce a spread on injection into the skin and that one make of commercial peptone

had a slight effect, whereas another was completely inactive. A number of lymphagogues of the second class, including concentrated solutions of sodium chloride and sugar, caused no appreciable spread. Leech extracts, on the other hand, had a marked effect and the analysis of this fact has provided the material for the present communication.

Material and Methods

Preparation of Leech Extract.—The medicinal leech was used as the source of the extract. In some tests the crop was partially emptied of the blood previously ingested by leaving the leech in contact with crystals of sodium chloride or with a concentrated salt solution. The bodies were washed thoroughly, first with tap water and then with distilled water. They were then cut into small pieces and passed once or twice through a Latapie masher. The resulting pulp was ground with sand and extracted with a volume of distilled water equal to six times the weight of the pulp. After centrifugation, the supernatant fluid was filtered through coarse paper. The total solid content of such extracts was about 12 to 18 mg. per cc.

The testicle extract used in some tests for comparison was prepared in the manner already described (2). The extraction was made with a volume of distilled water representing six times the weight of the pulp.

Determination of Spreading Power.—The power of an extract to spread was determined by measuring the area of diffusion 24 hours after the rabbit had been injected intradermally with the test solution mixed with India ink. The ink used as indicator was Higgins' India ink diluted 1:3 with water and filtered through a Berkefeld candle. The area of spread depends to a large extent on the individual permeability of the rabbit skin, which varies greatly from one animal to another. In an attempt to take these variations into account, the area of spread produced by the extract was divided by the area of spread produced by a control, in this case saline or Ringer's solution, giving an index of diffusion more or less independent of the individual permeability of the skin.

The relation which exists between the extent of the spread and the concentration of the factor in solution is not of a simple order. For instance, the area of spread normally produced by the undiluted extract may be reduced no more than one-half when a tenfold dilution of the extract is injected. This effect may be misleading when one has to compare the relative potency of different preparations obtained from the fractionation of an active extract. The difficulty may be partially obviated by testing repeatedly the solution at various dilutions and comparing the solutions giving equal spread. Such solutions may be assumed to contain an equal quantity of spreading factor.

Spreading Properties of Leech Extract.—Preliminary tests had indicated that leech extract increased the permeability of the skin

in a manner similar to that observed for testicle extract, but more markedly. The following experiments illustrate the comparative spreading power of the two agents.

For the tests 0.5 cc. of leech extract, prepared in the manner described above, and 0.25 cc. India ink indicator were mixed in the syringe and injected intracutaneously into the upper part of the flank of a rabbit. The first indication of the action of the solution was the lack of resistance as the injection proceeded, and the immediate flattening of the bleb thereafter. Dispersion of the ink particles through the dermis, a process which continued for several hours, was relatively rapid, the main direction of the spread being determined by gravity. 20 hours after the injection, the area of spread, calcu-

TABLE I

Effect of Leech and Testicle Extracts on the Permeability of the Rabbit Skin

Extract tested	Area of spread of 0.5 cc. extract plus 0.25 cc. India ink indicator	Area of spread of 0.5 cc. saline plus 0.25 cc. India ink indicator (control)	Ratio of active spread to spread of control
	sq. cm.	*sq. cm.*	
Leech.................................	120.6	5.0	24.1
Leech (1:50 dilution with saline).............	15.7	4.1	3.8
Rat testicle..............................	14.3	5.0	2.9
Bull testicle.............................	18.3	5.0	3.7

lated from four similar experiments, averaged 120.6 sq. cm. The spreading mixture was found to have reached the lower part of the abdomen, extending to the mid-ventral line, and as far back as the scrotum. The skin of the involved area was thickened and edematous.

Autopsy showed large amounts of a somewhat gelatinous fluid accumulated in the subcutaneous tissue of the lower part of the abdomen and of the testicle.

The results of four experiments are summarized in Table I. For comparison, the effects of the diluted leech factor, and of rat and bull testicle extracts, prepared in the same way and with the same proportion of fluid to tissue, are included in Table I. Fig. 1 illustrates the relative spreading power of leech and of bull testicle extracts. The

accumulation of fluid in the subcutaneous tissue, as a result of the injection of the leech extracts, would suggest that the leech factor, in at least one phase of its activity, affects the exchange of fluids in the living tissues.

The fact that the leech extract, diluted 50 times, is practically as active as the undiluted testicular extract would indicate that the spreading factor is 50 times more concentrated in the standard leech extract, or that the leech factor is a chemically different substance, endowed with a spreading power considerably greater than that of the factor from testicle. With this problem in view, the chemical properties of the leech factor have been investigated.

Chemical Properties of the Leech Spreading Factor

Experiments on Solubility.—The leech spreading factor is readily soluble in water. Aqueous extracts of different parts of the leech (head alone, or body separated from the head) were brought to pH 4.5 by the cautious addition of 0.1 N acetic acid. An abundant precipitate separated, and another precipitate was formed when the acid solution was neutralized by means of N NaOH. The results of the tests in the rabbit skin are given in Table II. It shows that, after removal of the two precipitates, no more than 10 to 20 per cent of the factor originally present was retained in the solution.

In the next experiment, an attempt was made to extract the factor directly with weak acid solutions. The pulp prepared by grinding the whole leech body was extracted with a volume of 0.1 N acetic acid equivalent to six times the weight of the tissue. A second lot was treated in the same way with 0.05 N acetic acid. The reaction of the two extracts after centrifugation and filtration was pH 4.0 and pH 4.4, respectively. The use of acid solution instead of water resulted in a marked reduction in the solid content of the extracts, which was 9.9 mg. per cc. for the 0.05 N acetic acid extract, against 18.2 mg. per cc. for the water extract. The spreading power of these preparations is given in Table II. It shows that the spreading factor is soluble in weak acid, but the water extract was regularly more active. However, the loss in spreading power seemed to be compensated by a parallel loss in inert matter. When the spreading power was compared in terms of dry weight, the acid extract appeared to be at least as active as the neutral extract, giving a spread of 9.6 sq. cm. per mg. of solids as compared to 7.6 sq. cm. for the water control.

The factor is not soluble in acetone. An aqueous extract was treated with acetone, and the precipitate collected and dried in air. The acetone filtrate was evaporated to dryness. Extracts prepared from the two fractions were tested for spreading power. The acetone soluble fraction was inactive, and no more than 40 per cent of the original activity was recovered from the precipitate. When the

results were calculated on the basis of dry weights, the extract from acetone pre-
cipitation was found to represent a purer product in that the spread obtained was
8.5 sq. cm. per mg. of solids, against 2.5 sq. cm. for the watery extract. The abso-
lute loss in spreading power suggests that the factor is partially denatured by
acetone.

Experiments on Filtration.—The leech factor readily passes a Berkefeld filter,
but is completely held by a collodion membrane which retains proteins. The

TABLE II

Solubility in Weak Acid of the Spreading Factor of Leech Extracts

Material extracted	Solvent	pH of extract	Treatment of extract	Spread in the rabbit skin		
				Area of spread of 0.5 cc. extract plus 0.25 cc. India ink indicator	Area of spread of 0.5 cc. saline plus 0.25 cc. India ink indicator	Ratio of active spread to spread of control
				sq. cm.	*sq. cm.*	
Leech head	Distilled water	6.9	—	96.5	5.3	18.2
	" "	6.9	Brought to pH 4.5 then neutralized	51.0	5.3	9.6
Leech body (head excluded)	Distilled water	7.1	—	37.4	3.4	11.0
	" "	7.1	Brought to pH 4.5 then neutralized	22.1	3.4	6.5
Entire leech	Distilled water	7.0	—	65.8	1.5	43.8
	N:10 acetic acid	4.0	Made neutral	40.7	1.5	27.1
Entire leech	Distilled water	7.0	—	139.7	5.5	25.4
	N:20 acetic acid	4.4	Made neutral	95.0	5.5	17.3

fresh extract of the leech proved to have little diffusible matter, as shown by the
fact that the dry weight was reduced only from 18.3 to 15.8 mg. per cc. after 5
hours dialysis in cellophane sacs, impermeable to proteins, on the shaking appa-
ratus of Northrop and Kunitz (4). This treatment caused no reduction in the
activity of the solution.

Inactivation by Heat.—A leech extract, prepared in the usual way, and diluted
1:2 with Ringer's solution, was heated by immersion in boiling water for 6 min-
utes. The resulting precipitate was removed and the clear supernatant fluid tested
at various dilutions in the rabbit skin. The results are shown in Table III.

The fact that the area of diffusion of the heated extract at the lowest dilution was not greater than that of the unheated extract, 50 times more diluted, indicates that no more than 2 per cent of the original spreading power was left after the heating.[1]

The fact that 90 to 98 per cent of the spreading power is lost upon heating a crude extract at 95°C. for 5 to 15 minutes, was demonstrated repeatedly in additional experiments. The spread produced by the heated extracts was usually delayed and progressed slowly, as in the case of material of low grade activity.

TABLE III

Effect of Heat on the Spreading Power of Leech Extracts

Test No.	Solutions tested	Dilutions tested	Solids in solution	Area of spread of 0.5 cc. saline plus 0.25 cc. India ink indicator	Non-heated extract		Extract heated at 95°C.		Physical changes on heating
					Area of spread of 0.5 cc. solution, plus 0.25 cc. India ink indicator	Ratio of active spread to spread of control	Area of spread of 0.5 cc. solution, plus 0.25 cc. India ink indicator	Ratio of active spread to spread of control	
			mg. per cc.	*sq. cm.*	*sq. cm.*		*sq. cm.*		
1	Leech extract (1:6)	1:2	5.90	6.7	66.7	10.0	40.7	6.0	Precipitate formed
	" "	1:20	0.60	6.7	58.8	9.0	23.5	3.8	" "
	" "	1:100	0.12	6.7	39.2	5.8	14.8	2.2	" "
2	Leech heads extract (1:20)		5.0	5.9	41.0	7.0	31.2	5.3	Remains clear

Extracts prepared from the leech head are usually purer than those prepared from the whole body. In the next experiment, leech heads were extracted with a volume of water equivalent to 20 times the weight of the tissue pulp. This extract, after centrifugation and filtration through paper, was immersed in boiling water for 15 minutes. In this case no precipitate developed to complicate the interpretation of the test. The results recorded in Table III show that, even in the absence of flocculation, the spreading power of the solution was considerably reduced by heat, indicating a direct action rather than a secondary inactivation by adsorption on a precipitate.

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[1] Incidentally, these results illustrate the point, discussed above, that the area of spread is not directly proportional to the concentration of the active factor.

Separation by Copper Sulfate.—In the repeated attempts to find a simple method for the purification of the leech factor, it was found that copper sulfate would precipitate large amounts of inert material without reducing the spreading power of the extract. A 0.5 per cent copper sulfate solution was added drop by drop to a water extract of leech heads until the reagent no longer produced further precipitation. After removal of the precipitate by filtration on paper, the clear solution was dialyzed against cold, distilled water on the shaking machine for 8 hours. Test showed that precipitation and dialysis had removed as much as 89.2 per cent of the solids of the original extract, the precipitation with copper having accounted for 60 per cent of the reduction. The final product, tested in the rabbit skin, showed a spreading power greater than that of the original extract. The relative

TABLE IV

Effect of Copper Sulfate on the Spreading Power of Leech Extracts

Solutions tested	Characters of extracts	Total solids	Spread in the rabbit skin		
			Area of spread of 0.5 cc. solution plus 0.25 cc. India ink indicator	Ratio of active spread to spread of control	Area of spread per mg. solids in solution
		mg. per cc.	*sq. cm.*		*sq. cm.*
Leech heads extract...........	Dark brown, turbid	14.7	93 0	17.0	6.3
Copper sulfate filtrate.........	Clear, colorless	5.5	110.4	20.0	20.0
Copper sulfate filtrate, dialyzed.	" "	1.6	94.6	17.1	59.0
Copper sulfate, 0.5 per cent solution (control)..........	—	—	5.5	—	—

spreading power, expressed in terms of dry matter in solution, was 6.3 sq. cm. per mg. solids for the untreated extract against 59.0 sq. cm. for the purified fraction. The results are shown in Table IV.

The increase in spreading power, after treatment with copper sulfate, is difficult to explain unless we assume that the action of the reagent dissociated inhibiting elements from the spreading factor, or that copper sulfate reacted with it to form a more active compound. A 0.5 per cent solution of copper sulfate had no apparent effect on skin permeability.

In the foregoing tests the leech factor was found to be soluble in water and in weak acid, but was precipitated by acetone. It did not

pass collodion or cellophane filters, which retain proteins. There is evidence that the factor is inactivated by heat. All active fractions from the leech gave a positive diazo reaction, similar in this respect to the active fractions separated from testicle (5). In these general properties the leech factor resembles the testicular factor which, in a recent study, was shown to present the characters of proteins. Elucidation of the chemical relationship which may exist between the spreading factor from the leech and that found in mammalian organs must await further experiments.

Relation between the Leech Anticoagulating and Spreading Factors

The anticoagulating properties of leech extracts have long been recognized (6). The occurrence of a spreading agent in the same extracts brought up the question of the possible identity of the two principles. The following experiments represent an attempt to dissociate the spreading from the anticoagulating factor.

As the anticoagulating factor is assumed to originate in the buccal cavity and the pharynx (7), extracts of the different parts of the leech body were prepared and tested for the two factors. The head, taking in the first 10 segments, was the source of one extract and the rest of the body of the other. In some tests the body was prepared before extraction by opening the crop and washing out the contents. Tests were also made of extracts prepared from the latter and from the gonads. The ovaries, spermatophores, and the nine pairs of testicles were extracted together. Before testing, the crop content was diluted up to six times its volume with water. In all cases the tissue extracts were prepared as described above with a volume of water representing six times the weight of the tissue. The results of the tests in the skin of rabbits are shown in Table V.

It will be seen from Table V that the greatest spread was obtained from the isolated head and gonads, these two fractions being about equal in effect. The spreading power exhibited by the crude body extract was practically lost when, prior to mincing and extraction, the organs were removed and the internal wall was washed with a stream of water. Washing the surface of the sex organs before extraction removed also much of the spreading factor. It can be inferred from this that the muscular sheet of the leech body contains no appreciable amount of the spreading factor, and that even an important part of that present in the extract of the unwashed gonads

may come from another source. It seems unlikely that surface washing would remove the factor if it existed in the tissue. Body cavities and the sex organs located within the adjoining segments may conceivably be contaminated by the content of the pharynx when the head is removed. According to these results, the anterior digestive tract would appear to be the main source of the spreading factor as well

TABLE V

Effects of Extracts from Different Parts of the Leech on Skin Permeability

Tissues extracted	Characters of the extracts	pH	Total solids	Area of spread of 0.5 cc. extract plus 0.25 cc. India ink indicator	Area of spread of 0.5 cc. saline plus 0.25 cc. India ink indicator (control)	Ratio of active spread to spread of control
			mg. per cc.	*sq. cm.*	*sq. cm.*	
Leech head............	Light brown or greenish in color; clear or opalescent	6 9–7.1	13.1	91.2	5.0	18.2
Leech gonads............	Light brown; opalescent	6.9–7.1	—	80.7	4.9	16.4
Leech gonads (washed)....	Light brown; opalescent	6.9–7.1	7 9	41.6	7.4	5.6
Leech body.............	Dark brown to red in color; turbid	7.1	14.7	66.9	4.9	13.6
Leech body (washed). ...	Dark brown to red in color; opalescent	7.1	8.0	21.0	7.4	2.8
Crop content (mainly ingested blood)..........	Dark red; clear	—	—	20.9	4.0	5.2

as that of the anticoagulating factor. Other methods have been sought to separate the two.

Spreading and Anticoagulating Power of Leech Extracts.—In the next series of experiments the leech extracts were tested for both spreading and anticoagulating properties. The anticoagulating power was determined by adding 0.5 cc. of leech extract to 2 cc. of fresh rabbit blood and recording the time of clotting.

As shown in Table VI, the extracts most active in retarding blood coagulation were also those endowed with the greatest spreading power. This parallelism was maintained when the leech extracts were heated by immersion in boiling water for 6 minutes. Purification by precipitation with copper sulfate and dialysis enhanced the spreading and anticoagulating properties of the extract. These results would

TABLE VI

Spreading and Anticoagulating Power of Leech Extracts

Test No.	Solutions tested	Dilution of original extract with saline	Area of spread of 0.5 cc. solution plus 0.25 cc. India ink indicator	Clotting time of 2 cc. fresh rabbit blood plus 0.5 cc. test solution
			sq. cm.	*min.*
1	*Extracts from various sources*			
	Leech head	1:20	61.6	100
	Leech body	1:20	44.0	14
	Leech gonads	1:20	32.3	9
	Saline (control)	—	6.6	5
2	Leech head	1:50	33.0	20
	Leech gonads (washed)	1:50	11.4	2
	Leech body (washed)	1:50	10.6	1
	Saline (control)	—	5.5	1
3	*Effect of heat on activity*			
	Leech head extract	1:600	39.2	33
	Leech head extract, heated	1:600	14.8	16
	Ringer's (control)	—	6.7	4
4	*Effect of copper purification*			
	Leech body extract	—	31.6	24
	Leech body extract after copper precipitation, and dialysis	—	37.0	Fluid after 12 hrs.
	Saline (control)	—		

seem to favor the view that both effects are produced by a single factor. This opinion is not supported by the results given in Table VII.

In this experiment, commercial hirudin (6, 7) and fresh leech extracts were compared, the stock solutions being adjusted to contain 5 mg. matter per cc. The anticoagulating power of the hirudin preparation appeared to be even superior to that of the fresh extract of the

same concentration, whereas its spreading power was low and was practically abolished by 1:10 dilution. This indicates that the spreading and anticoagulating properties may exist independently in the leech extracts.

From the foregoing experiments it appears that the spreading and anticoagulating factors have practically the same distribution in the body of the leech, originating for the most part in the anterior digestive tract.[2] It is still uncertain whether a single substance is endowed with both anticoagulating and spreading properties, although the

TABLE VII

Spreading and Anticoagulating Properties of Leech Extracts and of Commercial Hirudin

Solutions tested	Total solids in solution	Area of spread of 0.5 cc. solution plus 0.25 cc. India ink	Clotting time of 2 cc. fresh rabbit blood plus 0.5 cc. test solution				
			10 min.	15 min	2 hrs.	10 hrs.	20 hrs.
	mg. per cc.	sq. cm.					
Hirudin.................	5.0	19.5	‒	‒	‒	‒	+ Clot not con-
Leech head extract.........	5.0	54.5	‒	‒	‒	‒	+ tracted
Leech body extract..........	5.0	47.0	‒	‒	+		
Hirudin (1:10).............	0.5	6.5	‒	‒	+		
Leech head extract (1:10)....	0.5	27.1	‒	+			
Leech body extract (1:10)...	0.5	16.3	‒	+		Clot contracted	
Saline (control).............	‒	5.8	+				

existence of two factors appears to be probable. If two different factors are involved, they may be closely related chemically, a fact which would explain the difficulties encountered in their separation.

DISCUSSION

The equilibrium between tissue fluid and the blood is maintained by the capillary wall, acting as a semipermeable membrane, and the

[2] It is conceivable that the spreading factor is released together with the anticoagulating factor at the moment of the bite. Professor L. Delrez has brought to our attention the fact that the therapeutic application of leeches is sometimes attended by extensive suffusion of blood in the subcutaneous tissue.

interplay of physical forces which give rise to filtration, osmosis, and diffusion. The theory which would refer the formation of lymph to these factors, first formulated by Ludwig and Starling, has received the support of modern investigators, and the observations that have accumulated on the subject are best interpreted in terms of this hypothesis (8–10). Heidenhain made the observation that certain substances, when introduced into the circulation, had the power to increase the lymph flow from the thoracic duct without exerting any definite action on the blood pressure (3). At the same time he noted that the content of organic matter in the lymph was augmented. He held these observations to demonstrate a secretory function of the capillary wall. The interpretation of Heidenhain is not accepted now and the facts are taken to indicate that the permeability of the endo-thelium of the vessels has been increased. The intimate changes in-duced in the capillary wall by Heidenhain's lymphagogues of the first group, especially by extracts of leech and mussels, seem not to have been investigated. The effect of leech extract on lymph formation brings up the question whether the phenomenon of spread may be caused, at least in part, by a sudden increase in vascular permeability. An excess of fluid filtering through the capillary wall and flooding the tissue spaces may separate widely the components of the connective tissue where solutes or particles, if present, would be dispersed pas-sively. Against the view that a local increase in the permeability of the vessels is the only factor conditioning the spread, is the observa-tion that testicular (11) and leech extracts will spread, although to a lesser extent, in a fragment of skin separated from the body.

Azoproteins have been shown to spread when introduced into the dermis (12). Although the area of spread produced by the injection of leech extracts or azoproteins may be ultimately the same, there is evidence that the phenomenon is induced by a different mechanism in the two instances. During the active spread the skin injected with leech extract remains smooth and relatively flaccid, whereas that re-ceiving azoproteins becomes thickened and appears to be under ten-sion. The osmotic pressure of the azoproteins tested for spreading power was found to be considerably greater than that of the uncoupled proteins, which under the conditions of the experiments was negligible (12). The presence in the subcutaneous tissue of azoprotein solutions

of high osmotic pressure may cause large quantities of fluid to filter from the vessels into the tissue spaces, until an equilibrium is reached, without affecting necessarily the normal permeability of the blood capillary. As a result the tissue spaces would become distended temporarily by the excess of fluid, allowing a passive dispersion of the substances which may have been introduced along with the azoproteins. Further experiments may be necessary to test this possible mode of action of azoproteins.

SUMMARY

1. The injection of leech extracts into the skin increases its permeability, as shown both by the spread of fluid and of foreign particles through the dermis. The spread is followed some hours after the injection by more or less edema of the subcutaneous tissue.

2. A preliminary study of the chemical properties of the leech spreading factor indicates a similarity with the spreading factor prepared from testicle.

3. Attempts to separate the leech spreading and anticoagulating factors showed that the two have practically the same distribution in the leech body, extracts from the separated head being the most active.

4. It is undetermined whether two distinct factors are responsible for the spreading and anticoagulating properties of leech extracts. A chemical similarity is suggested by the fact that agents which affect the activity of one factor have a parallel effect on the other.

5. The mechanism of the spread produced by leech extracts and by other spreading agents is discussed.

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EXPLANATION OF PLATE 9

Fig. 1. Rabbit 9-99 (right side). The spread of India ink as influenced by: A, saline control; B, leech extract; C, bull testicle extract. The final measurements of the areas of spread were 6.6, 112.2, and 17.0 sq. cm., respectively. Under the action of the leech extract the ink particles spread extensively through the cutaneous tissue. Less than 24 hours after the injection, the mixtures of leech extract inoculated respectively on the two sides of the back had merged under the abdomen at the mid-ventral line. Ink particles were found accumulated along the abdomen as far back as the connective tissue of the scrotum. The subcutaneous tissue of the skin over the ventral surface of the abdomen was edematous.

(Claude: Spreading properties of leech extracts)

THE EFFECT OF DIET ON THE SUSCEPTIBILITY OF THE CANINE HEMATOPOIETIC FUNCTION TO DAMAGE BY AMIDOPYRINE

By D. K. MILLER, M.D., AND C. P. RHOADS, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

PLATE 10

The association of amidopyrine administration with the occurrence of acute agranulocytosis in human beings has been pointed out in a number of communications (1–4). However, attempts to confirm by experimentation the hypothesis of a toxic effect of amidopyrine on hematopoiesis have failed signally. Although in rare instances a decrease in the number of circulating granulocytes has been reported to follow the administration of amidopyrine to animals, such results have been so irregular as to throw serious doubt on the rôle of the drug as an inhibitor of blood formation.

Miller (5) and Climenko (6) have published evidence that in animals fed amidopyrine in solution, histological examination of the bone marrow discloses definite pathological changes even though no marked decrease in the numbers of granulocytes in the circulating blood is present. Miller and Rhoads (7), in a study of the hematological changes associated with acute black tongue in dogs, reported that the acute stomatitis was occasionally associated with a decreased number of granulocytes in the peripheral blood. Histological studies of bone marrow removed at the height of the granulopenia revealed a relative decrease of mature hematopoietic elements. The change observed presented certain similarities to the pathological alteration of the bone marrow in acute granulopenia of man.

In the case of acute stomatitis and granulopenia of the dog, the etiology is apparent; it is the feeding of a particular diet. In acute granulopenia of man the pathological changes are in certain respects similar to those of the dog, but the cause is obscure although amido-

pyrine administration appears to play some part. It seemed possible then, that a faulty dietary might well be one factor at least which increased the susceptibility to the toxic effect of amidopyrine. To substantiate this hypothesis experiment should prove that a dose of amidopyrine which is ineffective alone will cause a well defined disturbance of hematopoiesis when it is added to a diet which is not by itself causative of symptoms. Such experiments have been performed, but paradoxically, the resulting disturbance of the circulating blood cells manifested itself in the erythrocytes rather than in the leukocytes. The results are reported in this communication.

The experiments dealing with the effects of aromatic compounds on hematopoiesis have been reviewed recently in detail by Hamilton (8) and by Kracke (9) and need no discussion here. It suffices to state that the picture of progressive anemia, entirely similar to aplastic anemia in man, has not been encountered. Sudden and severe decreases in number of white cells have been the rule. This last fact led Kracke (1) to suggest that the rôle of amidopyrine in granulopenia was one similar to that of benzol in the blood dyscrasias which are due to that chemical.

The part played by diet in controlling the susceptibility of individuals to poisoning by aromatic compounds was suggested by studies of trinitrotoluol poisoning in Great Britain during the World War (10). Furthermore, Biberfeld (11) had observed that an inadequate dietary rendered rabbits susceptible to poisoning by amounts of methol which were non-toxic to animals fed a normal diet.

Methods

The animals employed were mongrel dogs of about 7 kilos in average weight. They were kept under standard conditions in individual cages with bedding of wood shavings.

The so called normal diet was one which is fed as routine; and empirically is known to be capable of maintaining dogs in good health over a period of several years. It is a mixture of cooked beef, bread, and dog biscuit. The black tongue diet was that described by Goldberger (12). It is known to cause acute black tongue when fed, without supplement, for a period of from 5 to 10 weeks. In an extensive study the feeding of this diet has never been known to cause symptoms in previously normal dogs after a shorter interval. The diet was composed of the following ingredients.

Articles of diet	Quantity	Nutrients		
		Protein	Fat	Carbohydrate
	gm.	*gm.*	*gm.*	*gm.*
Corn meal............................	400	33.6	18.8	296
California black-eyed peas................	50	10 7	0.7	30.4
Casein (purified)......................	60	52 0		
Cane sugar............................	32			32.0
Cottonseed oil........................	15		15.0	
Cod liver oil..........................	30		30.0	
Sodium chloride........	3			
Calcium carbonate......	10			
Total nutrients........................ ..		96.3	64.5	358.4
Nutrients per 1000 calories....................		40 1	26.9	149.3

The corn meal, peas, and casein were mixed and cooked for 2 hours in a steam cooker. The remaining ingredients were then added and thoroughly mixed. The dogs were fed daily and were allowed to eat as much as they chose.

Blood was taken from the jugular vein in a standard amount of potassium oxalate for routine examinations. Determinations of the number of erythrocytes and leukocytes were made in standard pipettes and counting chambers. The hemoglobin was estimated by the Sahli method, employing a glass standard. The Sahli tubes were carefully calibrated and checked at frequent intervals by the O_2-combining capacity method of Van Slyke (13).

The amidopyrine used was lot 1,433, manufactured by the H. A. Metz Laboratories, New York. It was administered by stomach tube in a 5 per cent solution in water. Mild heating was required to effect a solution of that strength.

General Results

The effect of the administration of 0.5 gm. of amidopyrine daily was tested on two groups of dogs. One group of 12 was fed the Goldberger black tongue diet and the other group of 3 received the normal diet. In the first group (Table I), severe ulcerative stomatitis and profound anemia appeared between the 2nd and 9th weeks of treatment in 9. Three dogs, Nos. 2, 11, and 12, failed to develop severe anemia. Anorexia, loss of weight, and diarrhea were marked features. No striking granulopenia was observed. The bone marrows of the animals which died revealed a suppression of hematopoietic maturation. In the control group of animals (Table II) which were subjected to the

same medication but were fed the normal diet, no symptoms resulted. The experiment was repeated, employing larger doses of amidopyrine (Tables III and IV).   Both stomatitis and anemia were severe in the animals fed the black tongue diet, but in those fed the normal diet only a mild anemia resulted.   No distinct granulopenia occurred at any time in any experimental animal although histological study of the bone marrows of the animals which died showed a well defined interference with maturation of both red and white cells.

Anemia

The fall of erythrocyte levels was rapid in those animals in which anemia occurred.   In the group fed amidopyrine plus the diet producing black tongue, the anemia was profound, the erythrocyte count in some instances reaching 50 per cent or less of the original level.   In general, the fall of hemoglobin levels paralleled the fall in erythrocytes, giving a color index which is approximately normal for the dog. Marked variation in size and shape of erythrocytes was a feature at the height of the anemia.   Reticulocyte counts were not done.   There was no increase in the bilirubin content of the serum as indicated by the icterus index.

Stomatitis

The stomatitis appearing in those dogs fed the black tongue diet supplemented by amidopyrine was somewhat similar to that seen when the diet alone was fed for a longer period of time.   The first manifestation was a marked dusky red coloration of the entire oral and pharyngeal mucous membrane.   After a short time, small, discrete ulcerations appeared, most frequently just anterior to the pharyngeal pillars.   These showed a base covered by yellow necrotic material and a raised, injected margin.   At this stage, salivation was severe.   In some instances the ulceration remained localized, and in others it progressed until multiple, coalescent lesions were present and were covered by a membrane of necrotic material.   The loss of tissue was more severe, though more localized, than that seen in most instances of black tongue due to feeding alone.

Pathological Changes of the Bone Marrow

In Figs. 1 and 2 are shown low and high power photomicrographs of the bone marrow of dog 3, which developed severe anemia and died following the combination of feeding the black tongue diet and the daily administration of 0.5 gm. of amidopyrine. Similar changes were seen in other animals which died.

The normal arrangement of femoral bone marrow in the dog is almost completely obliterated. Instead of well defined islands of orderly hematopoiesis, the cells are scattered irregularly throughout the marrow tissue (compare with Fig. 3 which shows normal canine, femoral marrow). There is a striking suppression of maturation, particularly as concerns the cells of the erythropoietic series. Normoblasts and late erythroblasts are almost completely absent. A very definite increase in young cells has taken place as shown by the presence of many undifferentiated forms which vary somewhat in size. The most common one is a small, round cell with a small amount of basophilic cytoplasm and a deeply staining nucleus containing a very large amount of chromatin. This is held to be the primitive erythropoietic cell of Sabin (14). Various modifications of the primitive cell are present; some have more cytoplasm and a looser arrangement of nuclear chromatin. All of the stages in development up to that of the erythroblast are represented but only a few examples of more mature red cells are at hand.

An interference with the maturation of the granulocytes is less marked but is definite, in spite of the absence of granulopenia in the circulating blood. Granular leukocytes and their precursors are present in reduced numbers in the marrow. Furthermore, they show well defined evidence of damage as indicated by pyknosis of nuclei, abnormal size and shape, and irregular staining. No interference with the megakaryocytes can be made out.

Experiment 1. Table I. Text-Fig. 1

In this experiment, 12 dogs fed the black tongue diet received 0.5 gm. of amidopyrine daily. The results can best be seen by examination of the protocol of an animal which is typical of the series. It should be noted that in this instance the oral lesions appeared in less

than 10 days after the experimental diet feeding was begun, whereas such changes have never been observed in this laboratory from diet feeding alone before the 5th week.

Dog 4.—

5–31–34. Black tongue diet feeding begun. Amidopyrine administration 0.5 gm. daily begun.

6–12–34. Multiple small shallow punched out ulcers have appeared on the buccal mucosa. These range from 0.2–0.5 cm. in diameter and show a yellow

TABLE I

High and Low Blood Levels in Dogs Receiving Goldberger Diet and Amidopyrine, 0.5 Gm. Daily

Dog No.	Before amidopyrine		Time on amido- pyrine	Time on diet	After amidopyrine		Remarks
	R.B.C. per c. mm.	Hb			R.B.C. per c. mm.	Hb	
	millions	*per cent*	*days*	*days*	*millions*	*per cent*	
1	560	62	8	8	249	39	Amidopyrine discontinued. Recovered
2	576	81	12	12	390	70	Died
3	672	111	29	29	175	30	"
4	669	102	33	33	356	62	"
5	558	86	35	55	293	44	Amidopyrine discontinued. Recovered
6	747	107	31	51	271	57	" "
7	704	93	30	50	288	55	" "
8	660	95	28	48	168	29	" "
9	589	87	19	33	291	41	" "
10	539	80	41	41	164	29	" "
11	459	90	57	57	399	73	—
12	783	103	30	40	527	92	—

base with a reddened margin. There is active salivation. The whole oral and pharyngeal mucous membrane is a deep and dusky red. There is fairly active diarrhea. The animal refuses food.

6–16–34. The ulcerated areas which have shown little change since 6–12 have now extended and show a tendency to coalesce. They are much deeper and show more necrotic tissue at the border.

6–20–34. The buccal and pharyngeal mucous membrane is almost replaced by an extensive coalescent, deep ulceration with gangrene of the submucous tissues. Salivation is very active and the animal appears ill. Diarrhea has been present every day. There is marked loss of weight, complete anorexia, and progressive

weakness. Amidopyrine is discontinued. From this time on there is a distinct tendency of the ulcerated areas to heal. Weakness and anorexia persist, however.

7-6-34. After a progressive increase in weakness the animal is found dead.
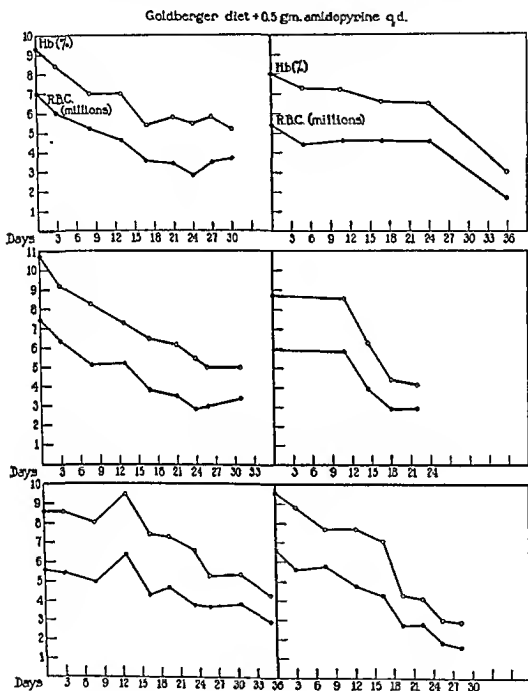
TEXT-FIG. 1

Autopsy.—An extensive gangrenous stomatitis is present. The marrow of the femur is a reddish yellow in color. No other gross lesions of the organs are seen. The microscopical changes are described under pathological alterations.

TABLE II

High and Low Blood Levels in Dogs Receiving Normal Diet and Amidopyrine,
0.5 Gm. Daily

Dog No.	Before amidopyrine		Time on amidopyrine	After amidopyrine		Remarks
	R.B.C. per c. mm.	Hb		R.B.C. per c. mm.	Hb	
	millions	*per cent*	*days*	*millions*	*per cent*	
1	644	86	36	468	86	Well
2	492	70	36	608	91	"
3	487	81	36	586	98	"

TEXT-FIG. 2

374
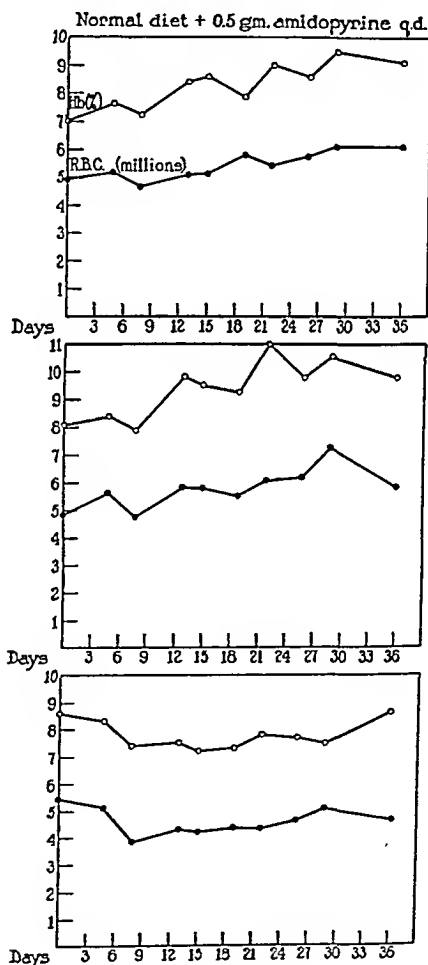
Experiment 2. Table II. Text-Fig. 2

This experiment served as a control on Experiment 1. 3 dogs fed the normal diet were given 0.5 gm. of amidopyrine daily. The two experiments were performed in parallel and the conditions differed in no detail other than the diet.

In no instance did either mouth lesions or blood dyscrasia occur in this group of animals. They remained in excellent health throughout the period of observation.

TABLE III

High and Low Blood Levels in Dogs Receiving 2 Gm. Amidopyrine Daily and Goldberger Diet

Dog No.	Before amidopyrine		Time on amido-pyrine	After amidopyrine		Remarks
	R.B.C. per c. mm.	Hb		R.B.C. per c. mm.	Hb	
	millions	*per cent*	*days*	*millions*	*per cent*	
1	6.63	105	19	1.55	26	Amidopyrine discontinued. Recovered
2	6.00	92	19	2.88	54	" "
3	6.29	95	16	2.89	43	Died after convulsion
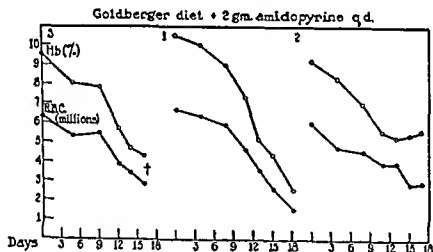
TEXT-FIG. 3

Experiment 3. Table III. Text-Fig. 3

Three dogs were fed the black tongue diet and received 2 gm. daily of amidopyrine. The results of the treatment are presented in Table III and in Text-fig. 3. All of the animals developed a severe grade of

anemia and one died. In the remaining 2, treatment was discontinued and recovery ensued. One striking discrepancy between this experiment and Experiment 1, in which only 0.5 gm. of amidopyrine was administered daily, was observed. The animals receiving a smaller amount of the drug all showed ulceration of the oral mucosa, whereas those given a larger amount did not manifest that change. No explanation is at hand for this difference.

Dog 3.—
4–23–34.   Black tongue diet administration begun.
5–9–34.   Amidopyrine, 2 gm. daily begun.
5–16–34.   A tonic and clonic convulsion occurs lasting several minutes following the amidopyrine administration. This is followed by a period of marked weakness of the extremities.
5–18–34.   Severe salivation follows the amidopyrine administration. No more convulsions have been observed.
5–26–34.   The animal has a severe anemia and is weak and pale. Amidopyrine administration is again followed by a convulsion. Later the dog is found dead.
*Autopsy.—*No abnormality of the organs except the bone marrow is found other than a very marked degree of pallor. The bone marrow appears redder and more cellular than normal to gross examination.

Experiment 4.   Table IV.   Text-Fig. 4

This experiment served as a control on Experiment 3. 5 animals were fed the normal diet and at the same time given 2 gm. of amidopyrine daily. The results are shown in Table IV. In contrast to the animals of Experiment 2, which received the same diet but only 0.5 gm. of amidopyrine daily, those given the larger amount of the drug did evince a definite fall in erythrocyte values. At no time, however, were mouth lesions or evidence of gastro-intestinal tract involvement, such as diarrhea, present. The animals continued to eat well and remained in relatively good health. The grade of anemia encountered in this group was not as severe as that seen in the animals fed the black tongue diet. In further experiments of this type similar results have been encountered. Whereas some depression of erythrocyte levels can be caused by the prolonged administration of large amounts of amidopyrine, it is irregular and of mild degree.

TABLE IV

*High and Low Blood Levels in Dogs Receiving Normal Diet and Amidopyrine,
2 Gm. Daily*

Dog No.	Before amidopyrine		Time on amido-pyrine	After amidopyrine		Remarks
	R.B.C. per c. mm	Hb		R.B.C. per c. mm	Hb	
	millions	*per cent*	*days*	*millions*	*per cent*	
4	6.29	89	15	3.59	69	Amidopyrine discontinued. Recovered
5	5.31	80	17	3.29	63	" "
6	5.07	75	9	2.82	43	" "
7	6.29	96	12	3.62	66	Died after convulsion
8	5 70	93	17	2.8t	48	Died, pneumonia
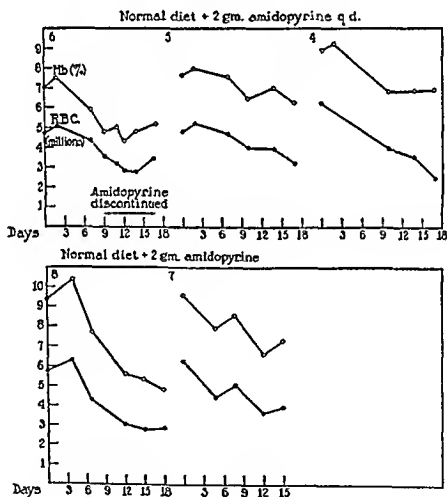
TEXT-FIG. 4
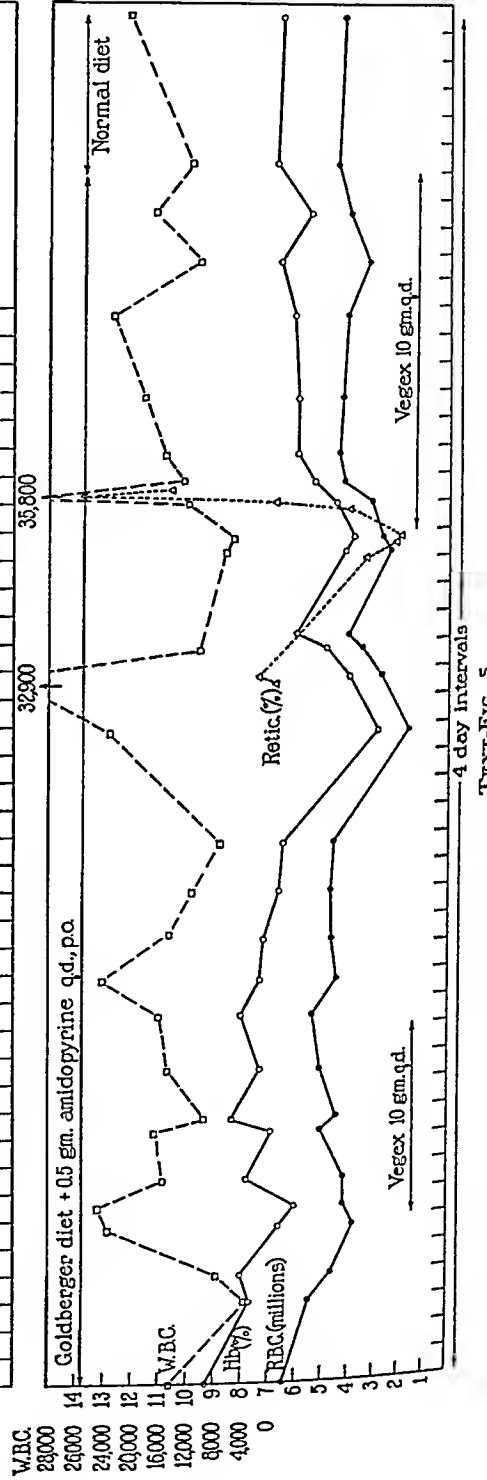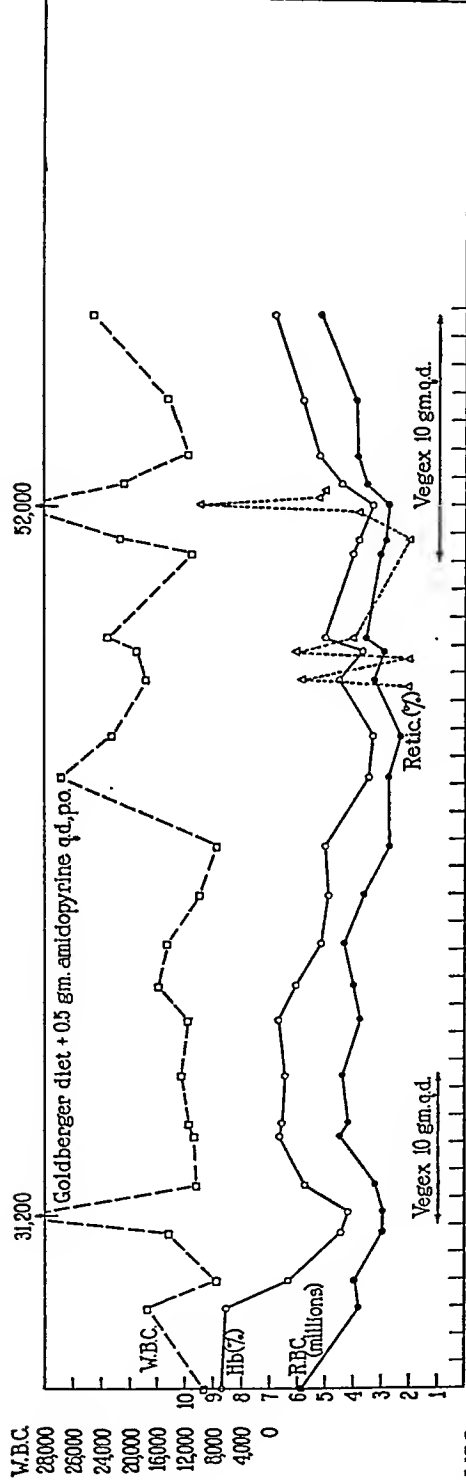
TEXT-FIG. 5.

Experiment 5. Text-Fig. 5

As a supplement to the studies which have been described, this experiment tested the effect of administering 0.5 gm. of amidopyrine daily to 2 dogs over long periods of the feeding of both good and bad diets.

The results are presented in Text-fig. 5. The administration of the drug during a period of normal diet feeding was without marked effect. When the black tongue diet was fed and the drug administration was continued, there was a pronounced decline of the blood values to low levels. When vegex, a commercial yeast preparation, was administered in amounts which were known to be effective in the prevention of black tongue, a remission of the anemia was effected. The blood was maintained at levels approaching the normal as long as the vegex supplement was continued, but fell when it was omitted. This was true in spite of the fact that the amidopyrine administration was continued throughout the course of the study.

DISCUSSION

In the experiments reported, anemia was brought about in dogs fed the black tongue diet by the administration of amounts of amidopyrine which were without effect when given to dogs fed a normal diet. When sufficient amounts of the drug were administered to dogs fed the normal diet, an anemia did result. It was not as severe as that caused by administering amidopyrine to animals on the inadequate diet and was not associated with stomatitis. Ulceration of the oral and pharyngeal mucous membrane was a striking feature when amidopyrine administration was combined with the inadequate diet. That the anemia and stomatitis were not due to the diet alone is clear from the fact that they occurred before they could be expected if only the special diet was fed. Moreover, they were more severe than are the changes of black tongue and were of a somewhat different character.

The absence of leukopenia was remarkable, particularly since it would have been expected, at least in a mild degree, from the histological appearance of the bone marrow. From this fact it would appear that the action of amidopyrine is neither exactly that of benzol nor of the black tongue diet alone. On the other hand, the toxic effect

on the bone marrow of the administration of an aromatic compound is so dependent upon such factors as dosage, diet, route of administration, and host susceptibility that it is difficult to prognosticate what the effect will be in a given instance.

The histological alterations of the bone marrow were of particular interest.   They appeared to be similar to those described in a case of benzol poisoning by Andersen (15) and also to those seen in several cases of aplastic anemia studied in this laboratory in which no history of exposure to benzol was available.

One aspect of the experiments deserves special consideration; the fact that the combination of amidopyrine administration and the diet feeding gave a pathological result not wholly dissimilar from that following a sufficiently long period of the diet feeding alone.   The stomatitis was similar to that of some cases of acute black tongue, though different in minor details from most.   Anemia of mild degree has been reported to occur irregularly in recurrent, chronic black tongue by Rhoads and Miller (16) and Spies (17).   A suppression of maturation of the hematopoietic elements of the bone marrow has been described in both acute and chronic black tongue by the same authors.   The suggestion may be advanced that the factor in the diet which makes it productive of black tongue is an aromatic compound or that the diet renders the body incapable of detoxifying some aromatic compound, either present in the diet or a product of intrinsic metabolic formation. Further experiments dealing with this subject are in progress.

SUMMARY AND CONCLUSION

1. By feeding dogs a black tongue diet and at the same time administering amidopyrine, acute stomatitis and anemia may be produced.

2. Both stomatitis and anemia occur some time before they could be expected to appear as result of the diet feeding alone.

3. The anemia is associated with suppression of maturation of the hematopoietic bone marrow elements.

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EXPLANATION OF PLATE 10

FIG. 1. Photomicrograph of the bone marrow of dog 2. The marrow is less cellular than normal and the degree of cell differentiation is reduced. Mallory's eosin and methylene blue.   ×250.

FIG. 2. Photomicrograph of the bone marrow of dog 2. Mallory's eosin and methylene blue.   ×1000.

FIG. 3. Photomicrograph of the normal canine femoral bone marrow. Mallory's eosin and methylene blue.   ×1000.
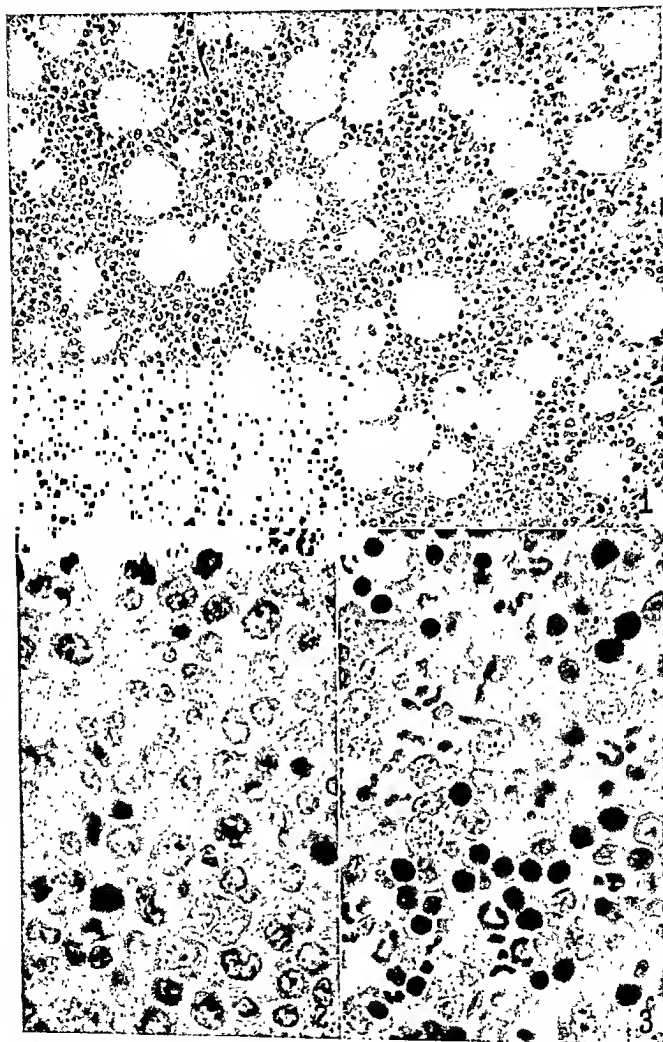
THE COMPOSITION OF SPECIFIC PRECIPITATES IN THE REGION OF ANTIGEN EXCESS

By SAUL MALKIEL AND WILLIAM C. BOYD, PH.D.

(*From Boston University School of Medicine, and Evans Memorial, Massachusetts Memorial Hospitals, Boston*)

There have been a number of studies of the composition of the precipitate formed in antigen-antibody systems in the neutral zone and the zone of antibody excess, and a quantitative theory (3, 4) of the precipitin reaction has been proposed. In these regions all, or by far the larger amount of the antigen is precipitated, and if we know the per cent of nitrogen in the antigen, we can calculate the composition of the precipitate from a simple determination of the total N of the precipitate. Heidelberger and Kendall (3, 4) have developed a method for determining the antigen in the supernatants of mixtures where all the antigen is not precipitated, which gives good results in part of the zone of antigen excess, but becomes unsatisfactory when the excess is large. It has also been shown (5) that by making use of the linear relation between the time of flocculation and the dilution of the antigen which holds with antibody excess, the antigen left in supernatants, or in fact any unknown concentration of antigen, can be rather accurately estimated. As yet this method has received little application.

Since the amount of antigen not precipitated in the region of antigen excess has thus proved difficult to measure, it is not surprising that few studies of the composition of the precipitate in this region have been made. The only one known to us which mapped out this region with any completeness is that of Heidelberger and Kendall (3), where the authors made use of a colored antigen obtained by coupling a dye to egg albumin (*cf.* 2). Part of the range in the case of the egg albumin system has been studied, but it was stated "... the inhibition zone data in the Ea-A system offer too many uncertainties to warrant treatment of this portion of the reaction range ..." A few points

have been obtained by Marrack and Smith (8).   No other data are
known to us where information is given on the location of any reference
point, such as the optimum or equivalence point (see below).   There-
fore it seemed desirable to investigate the composition of the precipi-
tate in this region to discover what regularities may obtain here in
the behavior of the precipitates, and to provide data for testing theo-
ries of serological reactions.   To supplement the study of Heidelberger
and Kendall (3) it seemed desirable to have data on a natural protein
and its antibody.

Attention has previously been called to the fact that the hem-
ocyanins are very satisfactory antigens for quantitative studies on the
precipitin reaction (6), because of their high antigenicity, and the
presence of a readily determined inorganic atom, or marker.   By
copper determinations on the precipitate formed the amount of
antigen therein contained is easily calculated, and by subtraction of
the antigen nitrogen from the total, that due to antibody is readily
found.   If we disregard small amounts of salts, lipoids, etc., this
determines the composition of the precipitate.   Since the analyses
are carried out upon the precipitate, the method is clearly independent
of the exact amount of antigen precipitated.

Methods

The analytical methods followed in the present study were the same employed
by Hooker and Boyd (6).

In order to have a reference point from which to measure the amount of anti-
body added to a given amount of antigen, the optimal proportions point, referred
to here as the optimum, was determined for each system, and precipitates pre-
pared using appropriate multiples and fractions of this amount of serum.   This
gave results more comparable than the use of arbitrary amounts of antiserum,
since it at least partly corrected for differences in strength of different sera.   The
equivalence point (mid-point of the equivalence zone) was also estimated in most
cases.   The actual amounts of the two reagents used depended upon the part of
the range being studied and upon the purpose for which the precipitate was to be
used, as those for copper determination had to be larger than those for total N.
Precipitates were kept first at 37° for 2 hours, then in the ice box overnight, or
longer in the case of large antigen excess.

The experiments were done in duplicate, i.e., four precipitates, two for copper
and two for total N, were prepared at each point of the range studied.   The
precipitates were washed with amounts of saline proportional to their size, ana-
lyzed, and the results matched according to relative magnitude, in other words,

the low copper paired with the low N, and so on. This gave better checks and seemed fully as defensible as any alternative procedure. The antigen nitrogen in the precipitate was calculated by multiplying the Cu value found by the ratio N/Cu in the hemocyanin. The analysis of separate precipitates, instead of aliquot portions of one larger precipitate, is attended with some loss in accuracy, as the precipitates are not always absolutely identical, but it has the advantage of simplicity and ease of manipulation.

Two purified hemocyanins, that of the horseshoe crab (*Limulus polyphemus*), and of a snail (*Viviparus malleatus*), were injected into rabbits, and two different bleedings, after 4 and 6 weeks, respectively, taken from each animal. These are designated by subscripts, the subscript ($_2$) referring to the later bleeding. The sera were passed through a Berkefeld filter.

RESULTS

The results of our analyses of precipitates, from the equivalence point to the largest antigen excess feasible with the amounts of serum at our disposal, are given in Tables I to VII. Our actual results have been recalculated to the basis of the amounts from 1 ml. of antiserum, and also to the basis of 1 mg. of antigen, to make the results comparable with each other. The results of the two methods of calculation are of course not identical, but the relation between antibody and antigen in each precipitate remains the same.

In the tables "fraction of optimal serum added" means that portion or multiple of the amount of serum found by the optimal proportions titration to be equivalent to 1 mg. of antigen, and "fraction of optimal antigen added" similarly means the portion of the amount of antigen found to be equivalent to 1 ml. of antiserum. These values are reciprocally related, that is, a mixture in which double the optimal amount of antigen has been added to a given amount of antiserum has the same composition as one in which to a given amount of antigen has been added one-half the optimal amount of serum. In this study we have considered the antiserum as the variable, since it has previously been maintained (7) that many features of the precipitin reaction can be explained by considering the primary process to be the surface coating of the antigen particles with more or less antibody. The completeness of the coating would then depend, among other factors, upon the relative amount of antibody available.

DISCUSSION

From the results in the tables it will be seen that the amount of precipitate obtainable from 1 ml. of antiserum increases with increasing amounts of antigen, reaches a maximum, and then declines, in agree-

TABLE I

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Viviparus Serum 926₁

Calculated to 1 ml. of serum				Calculated to 1 mg. of antigen				R
Fraction of optimal antigen added	Total N	Antigen N	Antibody N	Fraction of optimal serum added	Total N	Antigen N	Antibody N	
0.62	0.746	0.165	0.581	1.60	0.835	0.185	0.650	3.53
	0.773	0.166	0.607		0.865	0.185	0.680	3.66
0.71	0.820	0.189	0.632	1.40	0.801	0.184	0.617	3.34
	0.833	0.189	0.644		0.814	0.184	0.630	3.41
1.00	0.926	0.252	0.674	1.00	0.648	0.176	0.472	2.67
	0.940	0.272	0.668		0.660	0.192	0.468	2.45
1.25	1.053	0.312	0.741	0.80	0.592	0.176	0.416	2.37
	1.053	0.329	0.724		0.592	0.185	0.407	2.20
1.67	1.147	0.356	0.791	0.60	0.485	0.151	0.334	2.22
	1.150	0.373	0.777		0.486	0.158	0.328	2.07
2.50	1.220	0.429	0.791	0.40	0.341	0.120	0.221	1.85
	1.240	0.486	0.754		0.346	0.135	0.211	1.55
5.00	1.029	0.451	0.578	0.20	0.143	0.063	0.081	1.28
	1.035	0.590	0.445		0.144	0.082	0.062	0.75
10.0	0.650	0.363	0.287	0.10	0.045	0.025	0.020	0.79
	0.705	0.374	0.331		0.049	0.026	0.023	0.88

R stands for the ratio of antibody N to nitrogen N in the precipitate. For the meaning of other expressions used, see text. Equivalence point ratio for 926₁, 1.25 of optimal amount serum (per mg. of antigen).

ment with the data of other workers. The maximum did not in our experiments coincide with the optimum nor fall in the equivalence zone, but in general occurred far in the region of antigen excess.

Looking at the actual amounts of antigen and antibody precipitated, we see that each of these also passes through a maximum, but that the two maxima do not coincide, so that the precipitate maximum is the resultant of two tendencies. The maximum in the amount of antigen precipitated by 1 ml. of antiserum is not reached until rela-

TABLE II

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Viviparus Serum 926₂

Calculated to 1 ml. of serum				Calculated to 1 mg. of antigen				R
Fraction of optimal antigen added	Total N	Antigen N	Antibody N	Fraction of optimal serum added	Total N	Antigen N	Antibody N	
0.71	1.035	0.111	0.924	1.40	0.798	0.086	0.712	8.32
	1.058	0.170	0.888		0.816	0.131	0.685	5.23
1.00	1.170	0.209	0.961	1.00	0.651	0.116	0.535	4.60
	1.186	0.238	0 948		0 661	0.133	0.520	3.97
1.25	1.247	0.270	0.977	0.80	0.561	0.121	0.440	3.64
	1.252	0.313	0.939		0.563	0.141	0.422	3.00
1 67	1.376	0.341	1.035	0.60	0.462	0.115	0.347	3.04
	1.380	0.348	1.032		0.463	0.116	0.347	2.97
2.50	1.518	0.399	1.119	0.40	0.341	0.089	0.252	2.80
	1.527	0.494	1.033		0.344	0.111	0.233	2.09
5.00	1.362	0.480	0.882	0.20	0.152	0.054	0.098	1.83
	1.368	0.497	0.871		0.153	0.056	0.097	1.75
10.0	0.765	0.250	0.515	0.10	0.043	0.014	0.039	2.06
	0.865	0.339	0.526		0.049	0.019	0.030	1.55

Symbols as in Table I. Equivalence point = 2.00 optimal serum.

tively huge excesses (2- to 5-fold) of antigen have been added. From the right hand half of the tables it is seen that the amount of precipitate from a given amount of antigen is greater, the greater the amount of antiserum used (we know from other work that it approaches a maximal value for each system), and falls consistently as less antiserum is used, as do the amounts of the antibody and antigen individually.

We were primarily concerned in this work, however, to study the composition of the precipitate, as a function of the relative amounts of antibody and antigen mixed to produce it. One of us wrote previously (5): "... we need two relations, [1], $Ab/An = F(AB/AN)$, where the expression Ab/An means the ratio by weight of antibody

TABLE III

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Viviparus Serum 927[1]

Calculated to 1 ml. of serum				Calculated to 1 mg. of antigen				R
Fraction of optimal antigen added	Total N	Antigen N	Antibody N	Fraction of optimal serum added	Total N	Antigen N	Antibody N	
0.67	0.736	0.144	0.592	1.50	0.773	0.152	0.622	4.11
	0.778	0.172	0.606		0.817	0.181	0.636	3.53
1.00	0.890	0.200	0.690	1.00	0.625	0.142	0.483	3.45
	0.902	0.257	0.645		0.632	0.180	0.452	2.51
1.25	0.924	0.286	0.638	0.80	0.518	0.160	0.358	2.23
	0.935	0.302	0.633		0.525	0.170	0.355	2.10
1.67	1.027	0.287	0.740	0.60	0.432	0.121	0.311	2.58
	1.080	0.319	0.761		0.453	0.134	0.319	2.39
2.50	1.038	0.316	0.722	0.40	0.291	0.089	0.202	2.28
	1.150	0.378	0.772		0.322	0.106	0.216	2.04
5.00	1.220	0.488	0.732	0.20	0.170	0.068	0.102	1.50
	1.235	0.512	0.723		0.172	0.071	0.101	1.41
10.0	0.870	0.394	0.476	0.10	0.063	0.028	0.034	1.21
	1.190	0.466	0.724		0.086	0.034	0.052	1.55

Symbols as in Table I.

to antigen in the resulting precipitate, and AB/AN means the ratio of antibody to antigen mixed to produce this precipitate, and F, of course, is the sign of a function ..." (With the second relation proposed we are not concerned here.) It would seem that the present data establish the form which the above function takes in the region of antigen excess, at least for the systems studied

bere.   Reference to Fig. 1 will show that the ratio $R = Ab/An$ is in fact within limits of error a linear function of the fraction of optimal antibody used.   The figures for R unfortunately magnify somewhat, as do the results of indirect analysis, the experimental errors, since an error in the estimation of either Cu or N affects both the estimate of Ab and that of An.   But it would be difficult to find any other one type of curve which would fit the data better than a

TABLE IV

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Viviparus Serum 927₂

Calculated to 1 ml. of serum				Calculated to 1 mg. of antigen				R
Fraction of optimal antigen added	Total N	Antigen N	Antibody N	Fraction of optimal serum added	Total N	Antigen N	Antibody N	
0 71	0.567	0.160	0.407	1.40	0.555	0.156	0.399	2.55
	0.568	0.185	0.383		0.555	0.180	0.375	2.07
1.00	0 616	0.200	0.416	1.00	0.432	0 140	0.291	2.08
	0.620	0 236	0.384		0.435	0.166	0.269	1.63
1.25	0 639	0.225	0.414	0 80	0.359	0.127	0.232	1.84
	0.657	0 288	0.371		0.369	0.161	0.208	1.29
1 67	0.647	0.342	0 305	0.60	0.271	0.143	0.128	0.89
	0.669	0.410	0.259		0.281	0.173	0.108	0.63
5.00	0.433	0.337	0.096	0.20	0.060	0.047	0.013	0.28
	0.488	0.396	0.092		0.068	0.055	0.013	0 23

Symbols as in Table I.

straight line.   In the region of antibody excess the relation between R and the fraction of serum used is no longer linear, which was also found to be the case by other workers who investigated this part of the range.

This linear relation in the region of antigen excess between the ratio of antibody to antigen in the precipitate and the fraction of antibody used does not seem to have been commented on.   The data of Heidelberger and Kendall (3), plotted against the reciprocal of the amount

of antigen used, give also a straight line. Similar but somewhat more irregular results are obtained from the data of Heidelberger and Kendall on egg albumin (4), and the data of Marrack and Smith (8).

TABLE V

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Viviparus Serum 928$_I$

Calculated to 1 ml. of serum				Calculated to 1 mg. of antigen				R
Fraction of optimal antigen added	Total N	Antigen N	Antibody N	Fraction of optimal serum added	Total N	Antigen N	Antibody N	
0.63	0.722	0.152	0.570	1.60	0.808	0.171	0.637	3.75
	0.733	0.161	0.572		0.820	0.180	0.640	3.55
0.71	0.763	0.189	0.574	1.40	0.745	0.185	0.560	3.03
	0.772	0.189	0.583		0.755	0.185	0.570	3.08
1.00	0.891	0.238	0.653	1.00	0.623	0.167	0.456	2.74
	0.893	0.262	0.631		0.625	0.184	0.441	2.41
1.25	0.947	0.230	0.717	0.80	0.532	0.130	0.402	3.11
	1.025	0.353	0.672		0.575	0.198	0.377	1.91
1.67	1.066	0.458	0.608	0.60	0.447	0.192	0.255	1.33
	1.090	0.476	0.614		0.457	0.200	0.257	1.29
2.50	1.150	0.450	0.700	0.40	0.322	0.126	0.196	1.55
	1.196	0.450	0.746		0.355	0.126	0.209	1.66
5.00	1.192	0.672	0.520	0.20	0.167	0.094	0.073	0.77
	1.220	0.750	0.473		0.171	0.105	0.066	0.63
10.0	0.835	0.333	0.502	0.10	0.058	0.023	0.035	1.51
	0.883	0.383	0.500		0.062	0.027	0.035	1.31
20.0	0.586	0.322	0.264	0.05	0.020	0.011	0.009	0.81
	0.586	0.332	0.254		0.020	0.011	0.009	0.77

Symbols as in Table I. Equivalence point = 1.23 optimal serum.

These facts suggest that the mechanism of the reaction in this region may not prove to be so complicated after all.

The exact relation between R and the fraction of serum used seems to

depend partly upon the individual serum. If we write $R = a(AB/AN) + b$, the constant b, the intercept on the y axis, will have the significance of the limiting ratio of antibody to antigen as antibody is

TABLE VI

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Viviparus Serum 928₂

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Viviparus Serum 928[2]

Calculated to 1 ml. of serum				Calculated to 1 mg. of antigen				R
Fraction of optimal antigen added	Total N	Antigen N	Antibody N	Fraction of optimal serum added	Total N	Antigen N	Antibody N	
0.50	1.450	0.182	1.268	2.00	0.806	0.101	0.705	6.97
	1.496	0.214	1.282		0.832	0.119	0.713	6.00
0.63	1.546	0.239	1.307	1.60	0.695	0.107	0.588	5.46
	1.570	0.241	1.329		0 706	0 109	0.597	5.52
0 71	1.620	0.305	1.311	1.40	0.636	0.120	0.516	4.30
	1.619	0.306	1.313		0.636	0.120	0 516	4.30
1.00	1.804	0.380	1.424	1.00	0.501	0.105	0.386	3.75
	1.851	0.441	1.410		0.515	0.123	0.392	3.20
1.25	1.814	0.448	1.366	0.80	0.408	0.101	0.307	3.05
	1.919	0.455	1.464		0.432	0.102	0.330	3.22
1.67	1.980	0.546	1.434	0.60	0.333	0.092	0.241	2.63
	2.022	0.644	1.378		0.340	0.108	0.232	2.14
2.50	1.998	0.721	1.277	0.40	0.225	0.081	0.144	1.76
	2.017	0.763	1.254		0.453	0.172	0.282	1.65
5.00	1.759	0.576	1.183	0.20	0.098	0.032	0.066	2.05
	1.834	0.589	1.245		0.103	0.033	0.070	2.11
10.0	1.087	0.425	0.662	0.10	0.031	0.012	0.019	1.55
	1.088	0.452	0.636		0.061	0.013	0.018	1.41

Symbols as in Table I. Equivalence point = 2.16 optimal serum.

continuously decreased. Inspection of Fig. 1 and of Heidelberger and Kendall's data will show that this is in general not zero. It probably depends upon the properties of the antigen, such as molecular

weight and surface charge, but also presumably upon the character of the antibody, which is probably where the individuality of the animal enters.   The value of a, the slope of the curve, depends somewhat upon the individual serum, though the difference, in the present case, is not so very great.

Heidelberger and Kendall (3, 4) have found, in the region of antibody excess, a linear relation between R and the amount of antigen

TABLE VII

Antibody N, Antigen N, and Total N in Precipitates Made with Anti-Limulus Serum 779

Calculated to 1 ml. of serum				Calculated to 1 mg. of antigen				R
Fraction of optimal antigen added	Total N	Antigen N	Antibody N	Fraction of optimal serum added	Total N	Antigen N	Antibody N	
0.67	0.475	0.112	0.363	1.50	0.825	0.195	0.630	3.22
	0.473	0.101	0.372		0.821	0.175	0.646	3.68
1.00	0.532	0.157	0.375	1.00	0.617	0.182	0.435	2.39
	0.506	0.153	0.353		0.588	0.178	0.410	2.31
1.43	0.669	0.169	0.500	0.70	0.543	0.137	0.406	2.96
	0.561	0.169	0.392	.	0.455	0.137	0.318	2.32
2.00	0.486	0.251	0.235	0.50	0.283	0.146	0.137	0.94
	0.486	0.323	0.163		0.283	0.188	0.095	0.51
5.00	0.300	0.214	0.086	0.20	0.070	0.050	0.020	0.40
	0.301	0.245	0.056		0.070	0.057	0.013	0.23

Symbols as in Table I.   Equivalence point = 1.63 optimal serum.

N precipitated.   This relation does not hold in the region of antigen excess.

Heidelberger and Kendall have developed an equation which fits the data obtained by them and by other workers well in the equivalence zone and the region of antibody excess.   It does not account well for data for the region of antigen excess, as noted by these authors themselves, and as substitution in it of the present data will show.
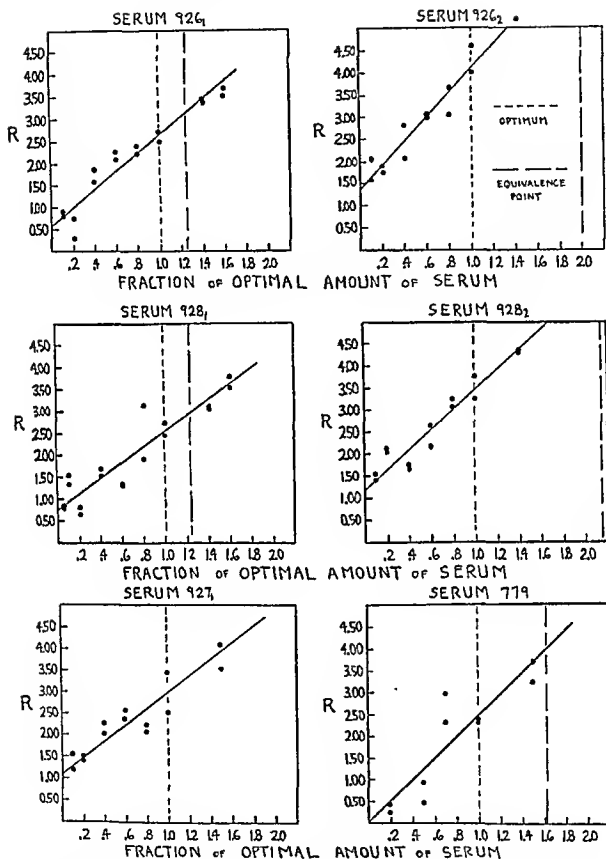
FIG. 1. Relation between ratio of antibody N to antigen N in precipitates (R), and the fraction of the optimal amount of antiserum added.

They give an equation for the region of excess antigen, which, in our symbols, is:

$$An = 2Ab'/R' - \frac{Ab'^2}{R'^2 An_a}$$

where An means the antigen nitrogen precipitated, Ab' the maximal antibody nitrogen precipitated, R' is the ratio of antibody to antigen at a reference point (where antigen first appears in excess), and An_a is the antigen nitrogen added. This is equivalent, for any given system to

$$An = k - \frac{k'}{An_a}$$

where k and k' are constants. This equation does not fit our data well either, and in fact it can be seen from its form that it will not fit data in the zone of partial inhibition, as the antigen nitrogen precipitated ought to go on increasing, approaching a maximum equal to k, but in fact our own data and those of Heidelberger and Kendall show that the antigen N precipitated eventually begins to fall off sharply. Thus it seems, as indicated by these authors themselves, that the theory of Heidelberger and Kendall does not apply well to data obtained with large antigen excess.

From Fig. 1 it will be noted that the equivalence point, as found by us for these sera, did not coincide with the optimum. The difference was not great with the earlier bleedings, but greatly increased with the later bleedings, a phenomenon probably related to the broadening of the equivalence zone observed by Heidelberger and Kendall.

If our curves are extrapolated to zero, i.e., to the point where no antibody at all is added, we obtain values for the limiting ratio of antibody to antigen. These values vary a good deal, and there is one anti-*Viviparus* serum, not shown on the graph, which gives zero for the y intercept, as does the anti-*Limulus* serum. The other sera give; 926_1, about 0.60, 926_2, about 1.40, 928_1, about 0.80, 928_2, about 1.20, 927_1, about 1.10. If we assume that the mean value of the limiting ratio is about 1.00, this would imply that the smallest amount of antibody which will combine to form a precipitate with one molecule of *Viviparus* hemocyanin is roughly an equal weight; assuming rabbit antibody to have a molecular weight of 138,000, and the hemocyanin a

molecular weight of 5,000,000, this would be about 36 molecules of antibody. Less antibody presumably forms a soluble compound with the antigen. It might be thought that the differences among our sera are partly due to antibody of better quality (more "avid") being formed in some animals, or to the production of antibodies to more than one antigenic determinant on the molecule.

It remains to be mentioned that the values of R presented here, at the optimum, and still less at the equivalence point, do not agree with the values predicted from the formula of Boyd and Hooker (1), if we assume *Viviparus* hemocyanin, like that of other snails studied by Svedberg, has a molecular weight of 5,000,000. Not only are the values too high, but the different sera vary considerably. Evidently the individuality of the animal is more important than was originally thought, and the relation of Boyd and Hooker only holds in a general way. We feel that on the whole, however, the available evidence justifies us in continuing to think that the ratio is importantly influenced by the molecular weight of the antigen. Heidelberger and Kendall (4) suggest that it also depends upon the relative numbers of reactive groupings in the antigen and antibody molecules. It seems to us that the quality (avidity) of antibody may be also an important factor.

SUMMARY

Data are given, for seven different antisera, for the composition of the specific precipitate as a function of the relative proportions of antiserum and antigen used. In the region of antigen excess, a linear relation is found between the ratio of antibody to antigen in the precipitate and the amount of antiserum used. The significance of these results, particularly in their bearing on theories of the precipitin reaction, is discussed.

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STUDIES ON PULMONARY EDEMA

I. The Consequences of Bilateral Cervical Vagotomy in the Rabbit[*][†]

By SIDNEY FARBER, M.D.

(From the Department of Pathology of The Harvard Medical School, and of The Children's and Infants' Hospital, Boston)

PLATES 11 TO 13

During the past several years, we have been interested in the finding of acute pulmonary edema in a number of necropsies performed on patients who suffered from central nervous system disturbances with involvement of the brain stem. Since, in these instances, the usual explanations for the pathogenesis of pulmonary edema were inadequate, an investigation of the conditions obtaining in these patients was made. Certain clinical and pathologic observations, which will be described at another time, pointed to involvement of the nuclei of the vagus nerves. An experimental approach to the problem was therefore directed along that line. This report is intended as an introduction to a study of pulmonary edema of this type.

Materials and Methods

Healthy rabbits varying in weight from 800 to 2500 gm. were selected. A variety of anesthetics was employed. The most satisfactory was local skin infiltration with novocaine (1 per cent). Urethane and various barbiturates were also used, and were administered either intravenously or intraperitoneally. Moon (1) has pointed out in this connection that large doses of sodium phenobarbital cause shock and pulmonary edema, and has warned against its use in experiments dealing with the lungs. Control studies were carried out in our experiments with the various drugs employed. In no case, with the minimal necessary amounts

used, was there observed any change in the lungs which could be confused with the pathologic alterations brought about by our experimental procedures. It may be mentioned that neither section of the depressor (aortic) nerves, which run as separate nerves in the rabbit, nor unilateral vagotomy (right or left) caused demonstrable changes in the lungs.

The vagus nerves were severed as low in the neck as was possible. It is evident that when bilateral cervical vagotomy is effected, the function of the recurrent laryngeal nerves is lost, and all vagal control below that level is abolished.

<div align="center">EXPERIMENTS</div>

Series 1.—

Survival Time.—Following bilateral cervical vagotomy rabbits of 800 to 2500 gm. in weight died usually in from 8 to 24 hours. In exceptional cases, a survival time of from 36 to 62 hours was observed. It should be emphasized that death invariably occurred after bilateral vagotomy. In general, the older and heavier animals lived longer than the younger animals. It was impossible, even after a large experience, to predict the survival time of a rabbit with any greater accuracy than within 1 to 2 hours.

Clinical Picture.—Soon after vagotomy, the respirations became slower and deeper. After 2 to 3 hours, the respiratory rate increased appreciably. Restlessness was noticeable. Soon the respiratory movements became rapid and labored, and distinct râles could be heard. A period of increasing excitement with marked dyspnea then ensued, followed within a few minutes by a crisis in the animal's condition. Frothy serous fluid poured from the nose and mouth. In a number of instances, the fluid was frankly hemorrhagic in character. An immediate and striking change in the animal's condition was now noted. The animal was once more quiet, and respiratory movements were slower and less labored. At intervals, smaller amounts of frothy fluid were expelled from the mouth and nose. It appeared likely that some of the fluid came from the mouth and not all from the upper respiratory tract. Episodes similar to the first crisis occurred once or twice more before the animals died. Death finally occurred after a short period of great excitement and dyspnea, with bubbles of frothy fluid in the mouth and nose. The animals appeared to be drowning in the fluid in the respiratory tract.

Postmortem Findings.—Necropsies were conducted in each case, and without delay in the majority of the experiments. The most striking changes were restricted to the upper respiratory tract and the lungs. No evidences of ascites or pleural or pericardial effusion were found.

The larynx, trachea, and bronchi were filled with frothy serous or serosanguineous fluid, which poured freely over the cutting board. Their mucosal surfaces were injected, but no purulent exudate in the gross was found. Occasionally, but not in all experiments, small amounts of thick mucus and vomitus were present in the upper respiratory tract. In no case, however, was obstruction to the upper

respiratory tract demonstrahle. The lungs were increased in size, and greatly increased in weight (Fig. 1). Rih markings were usually present on the posterior and lateral aspects of hoth lungs. The superficial lung tissue in hoth upper lohes was pink and overdistended. All remaining portions of hoth lungs were heavy, solid, and airless. The visceral pleura was stretched taut over these areas. On section, the lung surfaces in the involved portions were deep red to red-hlack in color, and completely consolidated. Frothy fluid could be expressed from the lung parenchyma. The vessels were engorged, and the tissues, in general, markedly congested. All hranches of the upper respiratory tract nnd the hronchial system appeared to he dilated, although a precise standard of comparison is not at hand. Moderate dilatation of the right auricle and right ventricle was present. Fluid hlood was usually found in the heart chamhers. If the autopsy was delayed for a short time after death, postmortem clots were generally found in the heart. Changes in the other organs have no hearing on this report, and will not he deserihed at this time.

On microscopic examination, no significant changes were noted in the heart. The mucosal surfaces of the larynx and trachea showed occasional ncute inflammatory changes. The submucosal veins and capillaries were enormously distended with hlood. In the lungs, variahle amounts of pneumonic exudate, consisting chiefly of neutrophiles and eosinophiles, were scattered throughout the involved areas, particularly in the lungs of those rahbits which survived longer than 18 hours. Occasional evidences of aspiration of food and vomitus were also present. Desquamated cells from the pharynx, mouth, and upper respiratory tract could he recognized in scattered areas. By far the most important change in the lungs, however, was the massive pulmonary edema, which involved not only the nir spaces and hronchioles, but the interstitial tissues as well (Fig. 2). The air spaces were filled with precipitated pale eosinophilic alhuminoid material, in which air bubbles were frequently entrapped. In some areas numerous hyaline memhranes[1] composed of deeply staining concentrated alhuminoid material lined the walls of alveoli and alveolar ducts (Fig. 3). In some instances, large areas of lung parenchyma were filled with red hlood cells and serum.

The interstitial tissues were more prominent than usual, and their components were separated as hy edema fluid. This was particularly true of the walls of the veins and arterioles. The perivascular lymphatics were greatly distended and filled with serum (Fig. 4). All veins nnd capillaries exhibited extreme degrees of

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[1] Wolbach (2) called nttention to the presence of hyaline membranes in the lungs of patients who died during the last influenza epidemic and explained the pathogenesis of the memhranes. Similar membranes may he found in a variety of conditions. Their presence is indicative of partial ohstruction to respiration caused hy fluid, plasma, exudate, or foreign materials of any kind in the lungs (Farher nnd Wilson, 3). In the experiments described above, the hyaline membranes are composed of albuminoid materials derived from the blood plasma.

distention (Fig. 5). The alveolar wall capillaries projected into alveolar spaces. The arterioles were also dilated, but were not as markedly involved as the veins.

The emphysematous areas, observed on gross examination, extended but a short distance beneath the pleura. Thinning of the walls and increase in size of the alveoli were the only noteworthy features in these areas.[2]

The chief gross and microscopic findings in rabbits which die after bilateral cervical vagotomy are therefore: (a) severe acute pulmonary edema and congestion; (b) variable amounts of acute bronchopneumonia; (c) evidences of aspiration of food and secretions; (d) terminal dilatation of the right side of the heart.

The postmortem findings in the lungs of rabbits which die after bilateral cervical vagotomy, are similar to those in certain diseases of the central nervous system, with involvement of the brain stem, such as the bulbar form of poliomyeloencephalitis. Certain clinical features, common to both the experimental animals and the human patients, strongly suggest a common pathogenesis.

There is a voluminous literature on the subject of bilateral cervical vagotomy. No review will be attempted here for adequate accounts are obtainable in the collected papers of Schiff, 1894 (4), the monograph of Frey, 1877 (5), and the more recent writings of Schafer (6). It is of interest that experiments concerned with the effects of double cervical vagotomy were mentioned by Galen, and Legallois (7), 1812, gives the history of such experiments from the time of Rufus of Ephesus, a Greek physician who lived in the reign of Trajan.

There is no agreement in the literature concerning the pathologic findings, the pathogenesis of the pulmonary changes, or the cause of death following bilateral cervical vagotomy. A vast amount of work based on ingenious experiments was carried out, particularly during the last century, and many observations which hold true today were made on the function of the vagus and its branches. The most impressive papers are those of Schiff. There are certain obvious explanations for the divergent conclusions defended by the most active workers: observations often were not extended long enough, secondary infections played too

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[2] The best results in the histologic demonstration of pulmonary edema were obtained when the lungs were fixed *in toto* in adequate amounts of 10 per cent formalin solution, or when thick sections of lung were so preserved. Thin sections were selected after several days. Placing of the bottles containing the fresh tissue in the incubator at a temperature of 52°C. for several hours, to aid in the precipitation of the edema fluid, is also of value, but is not a necessary procedure. Poor results were obtained when thin fresh sections of lung were fixed in Zenker's solution or in 10 per cent formalin solution.

important a rôle, and the age of the animal is of importance, since the less rigid laryngeal cartilages in very young animals permits laryngeal obstructions to occur more easily than in older animals. Furthermore, it is possible that not all types of animals behave in the same way; conditions in the cat and the dog may differ from those in the rabbit and guinea pig in important respects. No discussion of these differences will be attempted at this time. This paper is confined to the results of experiments on the rabbit.

The conclusions as given in the literature on this subject may be summarized. Death occurs after bilateral cervical vagotomy because of: (a) Aspiration pneumonia (vagus pneumonia); as a consequence of laryngeal paralysis, mouth secretions and food are aspirated causing an acute lobular pneumonia (Traube, 8, Frey, and others). (b) Slow asphyxia, secondary to laryngeal paralysis. With inspiration there is falling together of the thyro-arytenoid ligaments and the arytenoid cartilages (Legallois, Schafer). (c) Neuroparalytic pulmonary congestion, as a consequence of a loss of the tonic vasoconstrictor action of the vagosympathetic nerves (Schiff).

Our clinical and pathologic studies on the rabbit have disclosed the existence of a fourth factor to account for the pulmonary changes and death following bilateral cervical vagotomy. Asphyxia caused by the gradually increasing pulmonary edema serves to increase the severity of the pulmonary edema. The intense dyspnea, the frothy fluid in the upper air passages, and the hyaline membranes, entrapped air bubbles, and large amounts of fluid in the lungs on microscopic examination, are evidences of this. It is known that asphyxia causes dilatation of capillaries (Krogh, 9) and increases the permeability of capillaries (Landis, 10), factors which lead to pulmonary edema.

Since the pulmonary edema and congestion were of such marked degree, it appeared worthwhile to study the mechanism of their production with the factors mentioned above in mind. The first two conditions making for the fatal outcome result from the laryngeal paralysis that occurs when both vagus nerves are severed in the neck. In an extensive experience in the experimental study of slow asphyxia in animals, pulmonary edema of such severity as was observed in the present experiments was never encountered, an observation not in harmony with the theory of Schafer. The laryngeal factor can be excluded by the use of a tracheotomy tube. This method was used in a second series of experiments on rabbits.

Series 2.—

Rabbits were prepared as before, except that a cannula of suitable size was inserted into the trachea before a bilateral cervical vagotomy was performed. The trachea above the cannula was ligated, and in a number of experiments, the esophagus at the level of the tube was also ligated. Suitable control experiments were performed on rabbits equipped with tracheal cannulae. The vagus nerves were not sectioned. Such control animals were kept alive for a period of 24 hours longer than the usual survival time observed after bilateral cervical vagotomy. No pulmonary edema was found in postmortem examination performed when these animals were sacrificed.[3] It is of greatest importance to keep the tracheal cannula clean and free from obstruction. The patency of the cannula was always tested at necropsy.

In a series of rabbits prepared with tracheal cannulae, both vagus nerves were severed in the neck, as in series 1. All animals died. When litter mates were used, one with a cannula, and one without, the animal with a cannula survived approximately 20 per cent longer than the one without. Comparison of the survival time of the entire group in series 2 (with tracheal cannulae) with that of series 1, shows that if the factor of laryngeal paralysis is excluded, the survival time, which varied from 10 to 26 hours, is increased. The clinical picture was, in general, the same as in series 1, except for a somewhat later onset of dyspnea. It was of interest that even though the esophagus and the trachea above the cannula were tied, small amounts of fluid dropped from the nose and mouth during the last hour or two before death. An accumulation of secretions in the mouth is also a part of the picture produced by bilateral cervical vagotomy. The chief pathologic findings were acute pulmonary edema and congestion of about the same order of severity as in series 1. The gross and microscopic pictures were similar to those in the animals without cannulae. The chief differences were that no evidences of aspiration of food or mouth secretions, or of bronchopneumonia, were present.

DISCUSSION

We can conclude that the laryngeal paralysis, incidental to section of both vagus nerves in the neck, is not essential to the production of the severe pulmonary edema which follows bilateral cervical vagotomy in the rabbit. The observation that rabbits die after bilateral cervical vagotomy even when equipped with tracheal cannulae is in agreement with the reports of Schiff and others. The conclusions of a number of workers (Traube, Frey) to the contrary are not understandable in the light of our own experiments.

[3] The best method for causing immediate death was the injection into the cisterna magna of the rabbit of a few cubic centimeters of 1 per cent novocaine. Death occurs instantaneously. Confusing pulmonary changes are thus avoided.

In considering the results of the experiments performed in series 2, emphasis may be placed upon the production of severe pulmonary edema and congestion under conditions which permit the exclusion of infection, aspiration of foreign materials, and slow asphyxia. To denote the pathogenesis of this type of pulmonary edema, the term neuropathic pulmonary edema will be employed. Further studies concerning the pathogenesis of this type of pulmonary edema will be reported.

CONCLUSIONS

1. Bilateral cervical vagotomy in rabbits soon leads to death, usually within 8 to 24 hours.

2. Gradually increasing dyspnea, crises with expulsion of frothy, serous or sanguineous fluid from the mouth and nose, and terminal asphyxia are the important clinical features.

3. Postmortem examination reveals severe acute pulmonary edema and congestion, variable amounts of bronchopneumonia, and evidences of aspiration of food and secretions. This picture is similar to that found in the lungs in the bulbar form of poliomyelitis.

4. These changes are brought about by a combination of factors secondary to bilateral vagotomy: laryngeal paralysis (aspiration of food, slow asphyxia); loss of the vagal innervation of the lungs.

5. Laryngeal paralysis is not an essential factor in the production of severe pulmonary edema and death following bilateral cervical vagotomy.

6. To denote the pathogenesis of this type of edema, the term neuropathic pulmonary edema is employed.

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EXPLANATION OF PLATES

PLATE 11

FIG. 1. Photograph of lungs and opened trachea of rabbit which died 25 hours after bilateral vagotomy. The dark portions are consolidated because of intense congestion and edema. Superficial emphysema is present in the pale areas. The trachea is filled with fluid containing air bubbles.

Fɪɢ. 2. Photomicrograph of lung of rabbit which died as a result of bilateral cervical vagotomy. The bronchiole and alveolar spaces are filled with edema fluid in which numerous small air bubbles are entrapped. Note great dilatation of alveolar wall capillary near center of picture. Hematoxylin and eosin. Reduced from a magnification of 300 diameters.

Fɪɢ. 3. Photomicrograph of lung of rabbit which died as a result of bilateral cervical vagotomy. Note the hyaline membranes composed of materials derived from the blood plasma lining the walls of alveoli and alveolar ducts. The capillaries are greatly distended. Hematoxylin and eosin. Reduced from a magnification of 300 diameters.
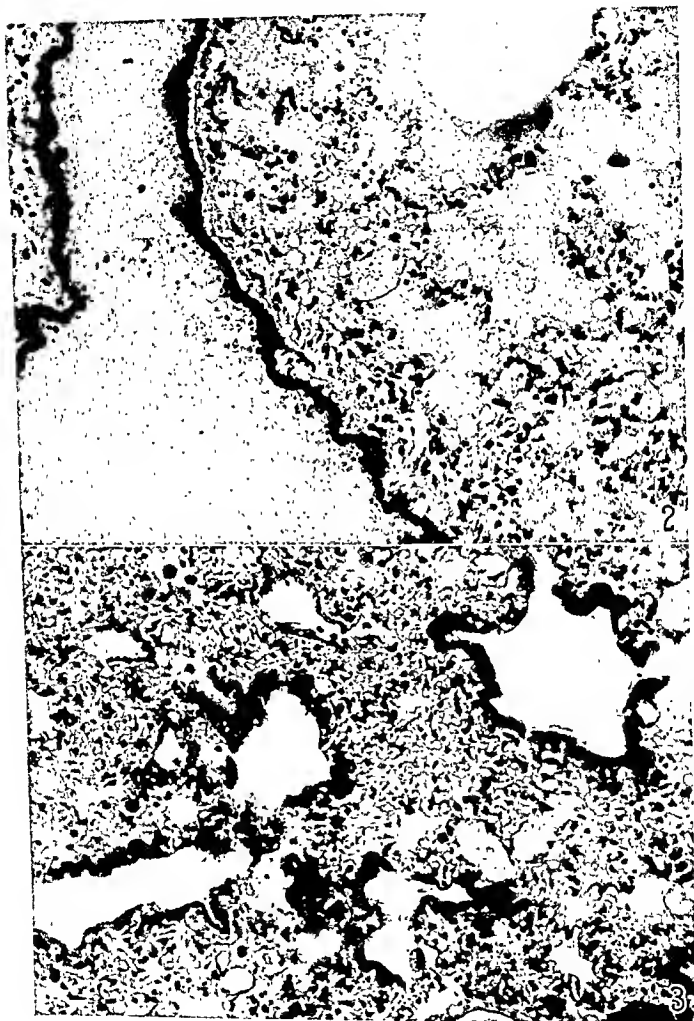
PLATE 13

FIG. 4. Photomicrograph of lung of rabbit which died as a result of bilateral cervical vagotomy. Note edema of periarterial tissues and dilatation of lymphatics which were filled with serum. The air spaces are filled with fluid and entrapped air. Hematoxylin and eosin. Reduced from a magnification of 250 diameters.

FIG. 5. Photomicrograph of lung of rabbit which died as a result of bilateral cervical vagotomy. Note great dilatation of a small vein. The surrounding air spaces are filled with edema fluid. Hematoxylin and eosin. Reduced from a magnification of 250 diameters.

STUDIES ON PULMONARY EDEMA

II. The Pathogenesis of Neuropathic Pulmonary Edema

By SIDNEY FARBER, M.D.

(From the Department of Pathology of The Harvard Medical School, and of The Children's and Infants' Hospital, Boston, and the J. F. Heymans Institute, University of Ghent, Ghent, Belgium)

Studies reported in an accompanying paper have shown that severe pulmonary edema, and death, are brought about in the rabbit by bilateral cervical vagotomy. Of the several factors which are of importance in producing the pulmonary changes under the experimental conditions employed, laryngeal paralysis was excluded as an essential part of the mechanism involved. There remains for consideration, the part played by other organs deprived of vagal innervation as a result of bilateral cervical vagotomy. The heart, as will be shown, can be excluded as a primary factor. The organs supplied by the subdiaphragmatic branches of the vagus nerves can be dismissed also. Recently Beazell and Ivy (1) destroyed all branches of the vagus below the diaphragm in the rabbit in a study concerning gastric ulcer. Their animals lived from 52 to 117 days after operation, a fact which, in view of our experimental findings, immediately excludes any important effect of the subdiaphragmatic branches on the lungs. We are led, then, to an investigation of the vagal innervation of the lungs in the pathogenesis of neuropathic pulmonary edema.

Material and Methods

Guinea pigs were used in these experiments. These animals varied in weight from 250 to 700 gm. The guinea pig was selected for this portion of the study because the survival time after bilateral cervical vagotomy is short (2½ to 4 hours for guinea pigs of 250 to 700 gm. in weight (2)). Furthermore, the time of death can be predicted with great accuracy after some experience. The clinical picture and the pathologic findings are almost exactly the same as in the rabbit, so that comparisons are permissible. The operative procedures were identical with those used in the experiments performed on rabbits. With the aid of urethane anes-

thesia the vagus nerves, unless otherwise stated, were severed low in the neck. One change in experimental procedure was employed in these experiments. After insertion of a tracheal cannula of suitable size, the cannula was connected to an apparatus for artificial respiration. Suitable control observations were made on a small series of guinea pigs with vagus nerves intact until the optimum conditions of continuous artificial respiration for the guinea pig were found. Such control experiments were continued for a period of 1 hour longer than the greatest survival time noted in a guinea pig after bilateral cervical vagotomy. Postmortem examination of these animals revealed no pulmonary edema. The lungs showed slight congestion, and a moderate deepening of the normal pink color. No changes in the lungs were observed which could be confused with the pathologic picture produced by section of the vagus nerves.

EXPERIMENTS

Series 1.—Under conditions of continuous artificial respiration and urethane anesthesia (injected intraperitoneally in amounts of 1.5 mg. per kilo of body weight) guinea pigs were subjected to bilateral cervical vagotomy. At intervals the frothy serous fluid which appeared at the mouth of the tracheal cannula was withdrawn by means of a 2 cc. syringe. The smallest possible amount of pressure was used in removing the fluid, no attempt being made to withdraw fluid lower down in the trachea and bronchial tree. Death occurred usually within $3\frac{1}{2}$ to 4 hours after vagotomy. Postmortem examination revealed the usual picture of massive consolidation caused by severe pulmonary edema and congestion.

The experiments in series 1 give further proof to supplement that obtained in the rabbit, that the factor of laryngeal paralysis is not essential to the production of acute pulmonary edema following bilateral cervical vagotomy.

Series 2.—The same experimental procedures were employed as in series 1, with one addition. Before the vagus nerves were cut in the neck, the sternum was raised to permit direct inspection of the heart and lungs. Care was taken to prevent loss of blood and shock. The animals were kept warm with the aid of a heating pad, and the contents of the thoracic cavity were kept warm and moist by the application of pieces of cotton saturated with hot normal saline solution. Suitable control experiments were first carried out under these conditions on guinea pigs with intact vagus nerves. Artificial respiration was continued for 5 hours, at which time the animals were still alive. The heart was beating regularly, and the lungs showed only slight congestion. No pulmonary edema was present.

Another group of guinea pigs was subjected to bilateral cervical vagotomy after the sternum had been elevated. About 30 minutes after vagotomy red petechiae

appeared in scattered areas over the lung surfaces. These petechiae gradually enlarged and finally coalesced to form confluent areas of red discoloration, and what later proved to be consolidation. The heart continued to beat regularly and vigorously until the last few minutes, when dilatation of the right side became apparent. The heart stopped beating, usually 3¾ to 4½ hours after the vagus nerves had been severed. Postmortem examination and histologic study revealed the usual picture of severe pulmonary edema and congestion.

The results of experiments in series 2 permit exclusion of possible alterations in the heart of any importance in the causation of acute pulmonary edema following bilateral vagotomy. These experiments also afforded an opportunity to study the gradual onset of the pulmonary edema produced under these experimental conditions.

Series 3.—The experiments in this series were designed to permit a direct approach to the rôle played by the innervation of the lungs in the production of neuropathic pulmonary edema. Exactly the same procedures were used as in series 2, with one exception. The vagus nerves were not severed. Instead, the innervation of the lungs was temporarily abolished by the application of small pieces of cotton saturated with 1 per cent novocaine solution both anteriorly and posteriorly to the roots of both lungs. Care was taken to prevent the spread of the solution to neighboring structures. The applications of novocaine were regularly renewed at intervals of ½ to 1 hour. Weak solutions of phenol were first tried, but were found unsatisfactory because of their injurious effect upon the tissues.

Under direct observation, lesions in the lungs developed as in series 2. The heart continued to beat regularly until a rather abrupt change occurred, usually about 4 to 4½ hours after the first application of novocaine had been made. Moderate dilatation of the right auricle and right ventricle was then observed. After a few minutes, the heart stopped, and artificial respiration was discontinued. In all important respects, both in the gross and microscopically, the lungs were duplicates of those of intact guinea pigs which died after bilateral cervical vagotomy. Severe pulmonary edema and congestion were the main features.

We may conclude from the experiments in series 3, that the loss of the innervation of the lungs is the essential factor brought about by bilateral cervical vagotomy in regard to the production of neuropathic pulmonary edema in the guinea pig. It is obvious that both the sympathetic and the vagal fibers are temporarily abolished by the experimental procedure employed in series 3. No attempt is made at this time to separate the action of the two types of pulmonary nerves.

DISCUSSION

The results of these experiments depend for explanation upon the innervation of the lungs.   The branches to the bronchi may be dismissed from consideration, since no evidence of bronchoconstriction was found.   The effects of vagotomy on the trachea and bronchi lie rather in the opposite direction, dilatation.   The histologic picture emphasizes the dilatation and engorgement of the pulmonary vessels, particularly the veins and capillaries.   Attention is therefore directed to the vasomotor control of the pulmonary vessels.   It is now generally agreed that such a vasomotor control does exist.

Adequate anatomic proof of the existence of nerve endings in the pulmonary vessels has been given by Karsner (3) and by Larsell (4).   Important contributions and critical summaries of the literature concerning the vasomotor control of the pulmonary vessels may be found in the writings of Luckhardt and Carlson (5), Wiggers (6), and Daly (7).   Luckhardt and Carlson not only demonstrated vagal vasoconstrictor fibers to the pulmonary arteries in the frog and the turtle, but observed that section of the vagosympathetic nerves leads to dilatation of the lung arteries on the same side.   These authors pointed out also that the injudicious use of atropine in the preparation of experimental animals was responsible for inconclusive or negative results of many previous workers.   We are indebted to Daly and his colleagues for most of the recent information concerning the vasomotor control of the pulmonary vessels.   Their experimental technique has been so perfected that many objections to the use of artificial preparations (perfused organ experiments) have been overcome (8–10).

Of direct bearing on the problem presented in this paper, is the status of the vasomotor control of the pulmonary vessels in the rabbit.   No references to the condition in the guinea pig are at hand.   Cavazzani, 1891 (11), noted a rise in pulmonary arterial pressure on stimulation of the cervical vagus nerves.   This was confirmed by von Euler, 1932 (12), who demonstrated, in addition, that such constriction was enhanced by eserine and suppressed by atropine.   von Euler found also that adrenalin (0.01 to 0.2 mg.) causes vasoconstriction in the rabbit lung.   This effect is opposed by ergotamine.

Ettinger and Hall (13) found that acetylcholine caused vasoconstriction in the rabbit lung.   This effect was prevented by atropine and increased by eserine. They also noted that a greater constrictor action could be effected by acetylcholine if the vessels were previously potentiated by adrenalin, barium chloride, or histamine.   Since there is only a feeble reaction of the pulmonary artery of the rabbit to adrenalin and to sympathetic stimulants, Ettinger and Hall suggest that the vagus or other parasympathetic fibers are mainly responsible for vasoconstriction.   They propose the hypothesis that parasympathetic fibers, probably vagal, are chiefly responsible for the degree of constriction of the pulmonary artery repre-

senting the ordinary activity of the rabbit, but that the parasympathetic acts synergically with the sympathetic, in that the latter must produce a certain grade of tonus before the former can act.

Sufficient evidence points to the existence of strong parasympathetic vasoconstrictor fibers and weak sympathetic vasoconstrictor fibers in the lung of the rabbit. The application of this conclusion to our problem is evident: Loss of the power of pulmonary vasoconstriction by section of the cervical vagus nerves deprives the pulmonary vessels of a mechanism necessary in the adjustment of pulmonary vascular dynamics. Assuming that some sympathetic vasoconstrictor control is left intact under these experimental conditions, only a weak degree of tone can be present, and apparently in an amount insufficient to meet the needs of altered, or even normal, pulmonary vascular dynamics.

It is interesting to recall here that the vascular changes secondary to loss of vagal vasomotor control of the pulmonary vessels (bilateral vagotomy) were termed neuroparalytic hyperemia of the lungs by Schiff (14) in 1847, a few years before the first demonstration of vasoconstrictor nerves in the cervical sympathetic. Schiff was anticipated in some ways by Legallois, 1812 (15), who stated that "there occurs without doubt a loss of tone within the lungs, a sort of paralysis since the various tissues supplied by the vagus are engorged with blood."

It is impossible to conclude from the literature on the vasomotor control of the pulmonary vessels what the status is in man. Nor is it possible to conclude with certainty, the exact status in the intact rabbit or guinea pig, since the best information has come from perfused organ or isolated vessel preparations. It does appear, however, from available evidence, that both the sympathetic and the vagal systems exert a control over the pulmonary and bronchial vessels. Section of the vagus nerves (which contain some sympathetic fibers) in the rabbit and guinea pig, or destruction of the vagal and sympathetic fibers supplying the lungs in the guinea pig does disturb that control to a serious extent, as is demonstrated in our experiments by the production of severe pulmonary edema which leads to death, when those experimental procedures are carried out. No attempt will be made at this time to define that disturbance of control more closely in terms of the exact inbalance of the pulmonary nerves effected by

our experimental methods.   Further investigation of these problems is indicated, and is being carried out.

Sufficient evidence from the experiments reported here, and from the literature in the related problem of the vasomotor control of the pulmonary vessels, is at hand to indicate that disturbances to the vasomotor control of the pulmonary vessels cause serious alterations in the dynamics of the pulmonary circulation, and in the integrity of the walls of the pulmonary vessels.   These alterations lead to the production of severe pulmonary edema and congestion, which are the immediate causes of death.   Preliminary studies in man indicate that in a number of conditions, the vasomotor control of the pulmonary vessels may be disturbed, either by central or peripheral disease, and that acute pulmonary edema is dependent upon such a disturbance. These studies suggest the pathogenesis of one type of acute pulmonary edema in man which we have termed neuropathic pulmonary edema.

CONCLUSIONS

1. Guinea pigs die shortly after bilateral cervical vagotomy, even when continuous artificial respiration effected through a tracheal cannula is carried out.   Death is caused by severe pulmonary edema and congestion.

2. Direct observation of the lungs after bilateral vagotomy demonstrates that pulmonary edema develops gradually and increases slowly in amount and severity.   Congestion precedes and accompanies the development of the edema.

3. Neuropathic pulmonary edema in the guinea pig is caused by disturbance to or abolition of the pulmonary vasomotor nerves.

4. The evidence obtained by experiments on animals suggests that neuropathic pulmonary edema in man is caused by disturbances, either central or peripheral, to the vasomotor control of the pulmonary vessels.

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PROPERTIES OF THE TYPE SPECIFIC PROTEINS OF ANTIPNEUMOCOCCUS SERA

I. The Mouse Protective Value of Type I Sera with Reference to the Precipitin Content

By KENNETH GOODNER, Ph.D., and FRANK L. HORSFALL, Jr., M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

Other factors being equal it might be expected that any specific immunological value of a given immune serum should be proportional to the amount of the specific antibody. For example it might be held that the effectiveness of a given antipneumococcus serum in protecting animals against pneumococcus infection could be judged by estimating the amount of protein specifically precipitable with the homologous capsular polysaccharide, that is, the amount of antibody protein (precipitin). This thesis is presumably tenable only if the injection of various animals with a single antigen gives rise to a single antibody, or to a mixture in fixed proportions of antibodies dominantly specific but differing in one or more chemical or immunological properties. Present evidence indicates that agglutination, precipitation, and complement fixation are due to the same substances in the serum. It has not as yet been demonstrated, however, that in a single serum there exists only one antibody substance specific for a given antigen.

This question is important from the practical as well as from the theoretical point of view. The clinician has generally held to the significance of tests for potency in which animals are used. This view has developed as a result of the fact that antipneumococcus sera of certain types afford excellent protection to the experimental animal and at the same time are effective therapeutic agents in lobar pneumonia. With other types of pneumococci, notably Type III, the immune sera may possess considerable antibody potency as judged by agglutination titer and yet be lacking in ability to protect animals against large numbers of pneumococci. Sera of these types have also proven less successful in the clinic.

On the other hand, it has long been recognized that evaluation by animal

2. At least three different dilutions of each serum are used in each test. The sera are progressively diluted $\times$ 2, the precise dilutions being determined by exploratory tests.

3. For the actual test at least 5 mice are injected with each combination of serum and culture. Each mouse receives 0.5 cc. culture dilution and 0.5 cc. of the appropriate serum dilution, these being mixed in the syringe and injected into the peritoneal cavity.

4. Mice surviving for 96 hours are considered as protected by the corresponding amount of serum.

Estimation of Protective Potencies

The most difficult feature of any method involving biological variables is the accurate evaluation of the results obtained. Various workers have adopted various end-points. Thus the minimum amount of serum which will protect 60 per cent of mice can be taken as the end-point. There are three objections to this and similar criteria. First, this method gives end-points which are as widely spaced as are the dilutions of serum, that is, only rough approximations of possible numerical values can be obtained. Secondly, the result is actually dependent on very few mice, those in the group receiving the particular protecting dilution and those of the group receiving the next lower amount of serum. Thirdly, results are not always sufficiently regular to permit an adequate determination of the end-point. Thus, with 5 or even 10 mice per dilution, two amounts of serum, one being twice the other, may result in the same percentage survival. Several attempts have been made to overcome this end-point difficulty. It seemed very desirable to obtain a sensitive numerical end-point based not on the small group of mice at any particular dilution level but rather one which would take into account the fate of all mice irrespective of the amount of serum.

Since the usual data, if plotted, give curves with much sharper slopes in the region of 50 per cent survival, it must be generally agreed that in this type of biological work this is the only logical end-point. After several different methods had been tried the so called Muench 50 per cent end-point accumulation method was adopted.

This method was devised by Dr. Hugo Muench of The Rockefeller Foundation and is extremely simple in application. It has been used in the analysis of the results of protection tests in yellow fever work by Lloyd, Theiler, and Ricci (13) and in studies on the titration of vaccine virus by Parker and Rivers (15). The latter authors discuss the validity of the method and the system used for obtaining the numerical end-point. In brief, the method is as follows: The survivals and deaths for each serum dilution are separated. The figures in each column are then accumulated, each column being added beginning at the smaller end. If the resulting figures are then plotted against serum dilutions the lines will cross at a point which represents the dilution of serum which should be used to bring

about a survival rate of 50 per cent. Actually it is unnecessary to plot these data since a simple calculation will provide the figure.

An illustration of the actual application of this method is provided in Table I. It will be noted that in this titration 10 mice have been used for each dilution of serum. The results in terms of percentage survival for each dilution are shown in column 4. When these figures are directly plotted a line connecting them forms a smooth curve and the 50 per cent end-point is easily determined. Results of this order are, however, very exceptional. In the fifth and sixth columns the survivals and deaths have been accumulated as described above. The percentage survival of each serum level has been calculated and is shown in column 7. On inspection it is obvious that the desired 50 per cent end-point lies between the serum dilutions 1~40 and 1~80. The exact point is obviously 23/46 or 0.50 of the distance between 1~40 and 1~80. Since the progression in serum dilutions is geometric it is necessary either to convert the basic dilution number (40 in this

TABLE I

Specimen Mouse Protection Test with Determination of 50 Per Cent End-Point by the Muench Accumulation Method

Serum dilution	Result			Accumulation			Calculated 50 per cent end-point
	Survived	Died	Survival	Survivals	Deaths	Survivals	
			per cent			*per cent*	
1–10	10	0	100	30	0	100	
1–20	9	1	90	20	1	95	
1–40	7	3	70	11	4	73	
1–80	3	7	30	4	11	27	1–56
1–160	1	9	10	1	20	5	

instance) into a log factor for multiplication, or much simpler in operation, to convert the factor 0.50 into a proportional factor by reference to a progression chart. The calculated end-point in this example is 1–56.

For the actual estimation of the potency of a given serum it is necessary to carry out protection titrations of the unknown serum and of the standard serum at the same time. This requirement tends to overcome the disadvantages of slight variations in the number of microorganisms in a culture from day to day. The end-point for each serum is then determined and by proportional calculation the potency of the unknown serum can be estimated. An example of actual titrations on the same serum at different times is given in Table II. Five independent titrations of an unknown serum have

TABLE II

Comparative Titrations for the Estimation of the Mouse Protective Potency of Lot AA Type I Antipneumococcus Horse Serum

Date	Standard Type I antipneumococcus horse serum (No. 425) 1000 units per cc.									Type I antipneumococcus horse serum lot AA									Estimated potency of serum AA units per cc.
	Dilutions	Results			Accumulation			Calculated 50 per cent end-point		Dilutions	Results			Accumulation			Calculated 50 per cent end-point		
		Survived	Died	Survival rate per cent	Survivors	Deaths	Survivals per cent				Survived	Died	Survival rate per cent	Survivors	Deaths	Survivals per cent			
Jan. 23	1–20	5	0	100	11	0	100	1–64		1–30	2	2	50	8	2	80	1–87		1360
	1–40	4	1	80	6	1	86			1–60	4	0	100	6	2	75			
	1–80	2	3	40	2	4	33			1–120	1	3	25	2	5	29			
	1–160	0	5	0	0	9	0			1–240	1	3	25	1	8	11			
Mar. 13	1–40	13	2	87	20	2	91	1–70		1–32	10	0	100	22	0	100	1–109		1560
	1–80	7	8	47	7	10	41			1–64	6	4	60	12	4	75			
	1–160	0	10	0	0	20	0			1–128	6	4	60	6	8	43			
										1–256	0	5	0	0	13	0			
Apr. 14	1–40	4	1	80	8	1	89	1–94		1–40	5	0	100	11	0	100	1–137		1460
	1–80	4	1	80	4	2	67			1–80	3	2	60	6	2	75			
	1–160	0	5	0	0	7	0			1–160	3	2	60	3	4	43			
Apr. 27	1–40	5	0	100	7	0	100	1–71		1–40	4	1	80	8	1	89	1–90		1270
	1–80	2	3	40	2	3	40			1–80	3	2	60	4	3	57			
	1–160	0	5	0	0	8	0			1–160	1	4	20	1	7	13			
May 6	1–40	3	2	60	3	2	60	1–45		1–40	2	3	40	6	3	67	1–62		1380
	1–80	0	5	0	0	7	0			1–80	2	3	40	4	6	40			
	1–160	0	5	0	0	12	0			1–160	2	3	40	2	9	18			

Mean number of mouse protective units per cc. of serum AA.................... 1405

been carried out simultaneously with titrations of the known or standard serum.

The data presented in Table II are fairly representative of the order of results generally obtained. Some of these titrations would present an impossible problem were it necessary to judge the end-points on a simple survival basis. Furthermore the results show the great advantages of simultaneous titrations of unknown and standard for although the actual end-points for each serum vary widely the variations are generally in the same direction for both sera at the same time. Thus when the actual unitage value is calculated one finds a rather close agreement considering the number of mice used in each titration. It is believed that the

TABLE III

Mouse Protective Potencies and Specifically Precipitable Antibody of Nine Type I Antipneumococcus Horse Sera

Group	Serum lot	Character	Protective units per cc.	Maximum specifically precipitable nitrogen	Protective units per mg. of specifically precipitable nitrogen	Mean protective ratio for each group
				mg. per cc.		
A	58	Raw, single	440	0 765	575	
	57	Raw, single	550	1.018	540	
	50	Raw, pooled	760	1 510	505	540
	14	Concentrated	3145	5.798	545	
B	49	Raw, pooled	500	0.645	775	
	59	Raw, single	870	1.081	805	
	60	Raw, single	1000	1 229	815	800
	AA	Raw, pooled	1405	1.844	760	
	13	Concentrated	6100	7.125	855	

mean value, 1405 protective units per cc., is as representative of the actual protective potency of the serum as any figure obtainable by any biological method. The probable error involved in titrations such as that illustrated with five separate determinations is about 10 per cent although no accurate estimate of this factor can be made without much longer series of titrations.

Antibody Content of Type I Antipneumococcus Horse Sera

With a measure of validity having been shown for the method of estimation of protective potencies and for the analysis of the results it is now possible to proceed to a comparison of the protective potency and the amount of specific antibody (precipitin) demonstrable *in vitro*.

These two forms of experimental analysis have been carried out on nine Type I antipneumococcus horse sera and the results are shown in Table III.

For the estimation of mouse protective potencies several titrations were carried out with each serum, the average number in this series being slightly over 4. The average number of mice per serum, exclusive of those involved in the simultaneous titration of the standard serum, was 73. These repeated titrations were necessary in order to secure numerical end-points with some degree of precision. Similarly the estimations of maximum specifically precipitable nitrogen represent data secured from complete precipitin curves, each point on which represents an average of determinations on two duplicate precipitates.

The nine antipneumococcus horse sera included in Table III fall into two groups dependent upon the number of mouse protective units per mg. of specifically precipitable nitrogen (hereinafter termed the *protective ratio*). Thus group A includes four sera, both raw and concentrated, with potencies ranging from 440 to 3145 units per cc. of serum. In the number of units per mg. of specifically precipitable nitrogen (protective ratios) however, these sera differ but slightly, the range being from 505 to 575 with a mean of 540 units per mg.

In contrast are the results with the sera of group B. Although the mouse protective potencies of these sera range from 500 to 6100 units per cc., the protective ratios are rather uniform ranging from 760 to 855 units per mg. with a mean value of 800.

It is perhaps merely fortuitous that the mean protective ratios of these two groups of Type I antipneumococcus horse sera stand in the approximate relation of 2:3. It does not seem likely however that the actual protective ratios of the various sera are matters of chance. The difference between the results of the two groups is considerably greater than the outside probability of error in either protection or nitrogen estimations.

Several possible sources of error do, however, exist. The validity of the mouse protection titrations has already been discussed, and while some allowance must be made for the probability of error in each instance, this possible deviation is not sufficiently great to account for the differences between the two groups. A second possibility is that the amounts of specifically precipitable nitrogen do not accurately represent the amounts of specific antibody. These determinations were, however, very carefully carried out and the results of extensive checking show that with any given lot of pneumococcus polysaccharide the maximum amount

of specifically precipitable nitrogen as determined by the standard procedure is a
fixed characteristic of a given serum. Whether one is warranted in assuming
that this precipitable nitrogen represents only specific antibody is another matter.
It has been shown by Heidelberger and Kendall (16), for example, that if Type II
capsular polysaccharide is added to a serum containing both Type I and Type II
antibodies, both will be found in the resulting precipitates. An example of a
quantitative experiment designed to determine the extent of this non-specific
precipitation is shown in Table IV. Type I capsular polysaccharide was added
to Type I and Type II sera separately and after these had been mixed. It will be
noted that while the addition of Type I capsular polysaccharide to the Type II
serum gave no precipitate the addition of the carbohydrate to the mixture of the
two sera gave a nitrogen result higher by 7.2 per cent than was obtained by the
addition of the polysaccharide to the Type I serum. This is regarded as a sig-

TABLE IV

*The Influence of Heterologous Antibodies on the Apparent Amount of Specifically
Precipitable Nitrogen*

Type I antipneumococcus horse serum	Type II antipneumococcus horse serum	Type I capsular polysaccharide	Precipitable nitrogen
cc.	*cc.*	*mg.*	*mg.*
1.0	—	0 25	1.490
1.0	1.0	0 25	1 597
—	1 0	0 25	0.000

Precipitation carried out at 4°C. according to the method of Heidelberger and
Kendall (9).

nificant increase but the amount is not great enough even under the conditions of
this experiment to affect seriously the general results shown in Table III.

Antibodies other than the type specific anticarbohydrate are present in prac-
tically every Type I antipneumococcus horse serum. Some of these, such as those
directed against the pneumococcus protein and the somatic carbohydrate (C sub-
stance) are present in amounts so small as to affect the results only to a very slight
extent even were a considerable proportion included in the immune precipitate.
Tests as to the monovalence of the immune horse sera were carried out and it was
found that only one, No. 58, gave any reaction with the capsular polysaccharides
of other pneumococcus types; this serum had antibodies which reacted with the
capsular polysaccharide of Type II Pneumococcus, although the reaction was
faint. It would therefore appear that the quantitative results have not been
seriously affected by non-specific reactions.

Since there appears to be no obvious experimental error sufficient
to account for the observed differences between the two groups of

immune horse sera another explanation must be sought. In the absence of definitive evidence any explanation must, however, be of a hypothetical nature.

As a first possibility it may be that certain of these sera contain substances other than the specific antibody which largely condition the *in vivo* efficacy of these antibodies. From the results of studies on the relation of lipids to immunological reactions, one might hold that an abnormal lipid pattern would tend to inhibit the protective action of the serum (17). This possibility seems somewhat unlikely, however, in view of the fact that each group included both pooled sera and sera from single bleedings. Furthermore if some inhibitor were present in the sera of group A it would seem unlikely that the amount would be so constant with respect to the amount of precipitable antibody as to give the con-

TABLE V

Mouse Protective Potencies and Specifically Precipitable Antibody of Seven Type I Antipneumococcus Rabbit Sera

Serum lot	Protective units per cc.	Maximum specifically precipitable nitrogen	Protective units per mg. of specifically precipitable nitrogen	Mean protective ratio
		mg. per cc.		
22	1070	0.938	1140	
28	1130	0.986	1145	
27	1190	1.108	1075	
A7	1270	1.128	1125	1170
26	1390	1.116	1245	
25	1830	1.533	1195	
24	1910	1.510	1265	

All lots represent pooled bleedings and the sera have not been concentrated.

sistent results obtained. If the inhibitor were intimately and quantitatively associated with the antibody molecules this hypothesis might be tenable.

A second explanation is that certain horses may produce antibodies having greater protective values. Here again the matter of sera from single and pooled bleedings would seem to make this possibility slight.

A third possibility is that the antibodies in a given immune horse serum, although reacting with the specific polysaccharide, may in fact represent a series of substances possessing different avidities, different degrees of specificity, different protective values, and perhaps even different chemical properties. It is possible that an immune serum may contain a mixture of specifically reactive antibodies varying in one or more of these properties.

Antibody Content of Antipneumococcus Rabbit Sera

Determinations of protective potencies and of maximum amounts of specifically precipitable nitrogen have been carried out with seven Type I antipneumococcus rabbit sera. The results of these determinations are presented in Table V.

The estimations of protective potencies are based on an average of 4.7 titrations per serum, with an average number of 77 mice per serum, exclusive of those involved in the simultaneous titration of the standard serum.

It will be noted that the protective values of the immune rabbit sera range from 1070 to 1910 units per cc. but that the number of protective units per mg. of specifically precipitable nitrogen is relatively constant, averaging 1170. Thus Type I antipneumococcus rabbit sera appear to be uniform in their protective ratios. This result stands in contrast to those obtained with antipneumococcus horse sera.

There is reason therefore to believe that in the case of immune rabbit serum the protective potency can be directly estimated from determinations of the maximum amount of specifically precipitable protein. This, however, is true only under certain definite conditions. It has been clearly pointed out by Heidelberger, Kendall, and Scherp (8) that various preparations of capsular polysaccharide vary in their capacity to precipitate the antibodies of antipneumococcus rabbit serum. This variation is so great that it is necessary to test each polysaccharide preparation. It is believed, however, that if a given polysaccharide preparation is standardized against one serum and if this serum has been fully appraised in a mouse protection test, one should be able to standardize all other immune rabbit sera of the same type on the basis of the known precipitating properties of the given polysaccharide. It is known, for example, that the lot of polysaccharide used in these experiments will precipitate approximately 92 per cent of the specific protective antibody from a given antipneumococcus rabbit serum.

The general differences between the protective ratios of immune horse and immune rabbit sera can perhaps be partially explained. With Bauer (18) it has been shown by ultrafiltration that the horse antibodies are in general larger than those of immune rabbit serum. Heidelberger and Pedersen (19) have demonstrated similar differences by the use of the ultracentrifuge. Although in certain details the results by the two methods are not in complete accord, there is now little doubt but that a difference in size does exist. If the rabbit antibody is actually smaller than that of the horse there should be in a given mass of rabbit antibody a much greater reactive surface and therefore one would expect that a

greater amount of carbohydrate could be bound in proportion to the amount of antibody. At the present time it is not possible to conclude that this explanation accounts for the observed differences in protective ratios, but the facts are suggestive.

SUMMARY

The ability to carry out with some measure of precision mouse protection tests for the estimation of potency of antipneumococcus sera has made possible the correlation of the protective potency with the amount of specifically precipitable protein. With antipneumococcus rabbit sera these protective ratios are relatively constant and higher than those with immune horse serum. Type I antipneumococcus horse sera, on the other hand, show no such constancy but fall into two groups; and there is as yet no simple method for determining to which group a serum belongs.

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PROPERTIES OF THE TYPE SPECIFIC PROTEINS OF ANTIPNEUMOCOCCUS SERA

II. Immunological Fractionation of Type I Antipneumococcus Horse and Rabbit Sera

By KENNETH GOODNER, Ph.D., and FRANK L. HORSFALL, Jr., M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

In the preceding paper of this series (1) it was reported that the amount of specific antibody precipitable by the capsular polysaccharide is related to the mouse protective value of Type I antipneumococcus serum only under certain well defined conditions. Thus with Type I antipneumococcus rabbit serum there was a direct proportionality between the two determined values. The protective ratio for immune rabbit serum, that is, the number of protective units per mg. of specifically precipitable nitrogen, was, however, higher than that for Type I antipneumococcus horse serum. Moreover, it was found that with the latter there existed at least two different protective ratios. Thus antipneumococcus rabbit sera average 1170 mouse protective units per mg. of specifically precipitable nitrogen, whereas horse sera fall into two groups having average values of 540 and 800 units per mg., respectively.

Several hypotheses were advanced as possible explanations for the differences in the protective ratios of different lots of antipneumococcus horse sera. It was reasoned that if, according to the generally accepted thesis, the horse had formed only one antibody specific for the pneumococcus capsular polysaccharide, the protective ratios of the sera might differ because of certain secondary factors. It is known, for example, that the protective action of antipneumococcus horse serum can be readily modified by admixture with certain lipids in very small amounts (2). The consistency of the results within the groups would necessitate a degree of uniformity in distribution of secondary substances which would seem to render this possibility somewhat unlikely.

It was also suggested as a possibility that more than one substance in the immune horse serum might possess the capacity of reacting

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with the capsular polysaccharide but that each of these possible anti-
bodies might possess characteristically different capacities to confer
passive protection on mice.   It was held that if this were correct
immune horse sera might represent equilibrated mixtures of anti-
bodies, dominantly type specific, but varying in degree as to protective
action.   These various hypotheses are somewhat difficult of experi-
mental proof but two forms of experimentation have been devised to
determine if all type specific antibodies in a given antipneumococcus
serum possess the same capacity to confer protection upon mice.   The
first approach is based on the fractional precipitation of the antibody
from serum by the addition of various amounts of specific polysac-
charide.   From analyses of the amount of protein specifically pre-
cipitated and of the amount of protective antibody remaining in the
absorbed serum, it is possible to calculate the protective value of each
fraction of antibody precipitated.   The data derived from experi-
ments of this order form the subject matter of the present paper.

A second and more direct approach has also been employed, namely,
the attempt to isolate antibody proteins in more or less pure form with
analyses of the protective values of the variously isolated fractions.
The results of this study will be presented in a subsequent paper.

EXPERIMENTAL

Materials and Methods.—A detailed description of the immune sera, of the
capsular polysaccharide preparation, and of the general methods of procedure
used in the present study has been given in the preceding paper (1).   In brief, the
method for the determination of the amount of specifically precipitable nitrogen
in the immune precipitates is that proposed by Heidelberger and Kendall (3).
Precipitates are prepared in the cold and after 18 hours at 4°C. are carefully
washed twice with ice-cold saline.   Protein precipitated is determined as nitro-
gen.   For mouse protection tests the method of Kirkbride, Hendry, and Mur-
dick (4) has been used and the results analyzed by the method of Muench (5).
Experimental evidence supporting the accuracy of the technical procedures
employed has been given in the preceding paper (1).

Immunological Fractionation of Antipneumococcus Horse Serum

Heidelberger and Kendall in a series of papers have demonstrated
the quantitative interrelationships between the amount of capsular
polysaccharide added to a specific immune serum and the resultant
amount of immune precipitate (6).   It is possible to remove a definite

and predictable quantity of the specific antibody by the addition of a known amount of polysaccharide, and to estimate the amount of antibody remaining in solution either by difference or by direct determination. At the same time it is possible to estimate the amount of protective antibody remaining in the absorbed serum by the tests and analytical procedures previously described (1). If a series of partial absorptions is carried out and the supernatant fluids so remaining after each precipitation are analyzed, it is possible to calculate by difference the protective potency of the antibody removed by each addition of specific polysaccharide.

In the first experiment Type I antipneumococcus horse serum, lot AA was used. By repeated mouse protection titration against a standard serum the test serum has been found to contain approximately 1405 protective units per cc.

To each of a large series of tubes was added exactly 2.0 cc. of a 1:2 dilution of sediment-free serum and the tubes were then chilled. Various amounts of chilled capsular polysaccharide solution were then added as indicated in Table I. The volumes were then made up precisely to 4.0 cc. with cold saline. After 18 hours at 4°C. all tubes were centrifuged for 1 hour in the cold. The supernatant fluids were then decanted and those from duplicate tubes were pooled. The precipitates were carefully washed twice with ice-cold saline and the precipitated protein determined as nitrogen. The analyses, carried out in quadruplicate, therefore represent the amount of nitrogen specifically precipitable from 1 cc. of serum by the indicated amount of polysaccharide.

Simultaneous mouse protection tests were next carried out with the supernatant solutions which represented exactly a 1–4 dilution of the original serum. The number of mouse protective units in the supernatant fluids was calculated by reference to the unitage potency of the unadsorbed serum as previously determined by simultaneous titrations against a standard.

Table I shows the amounts of nitrogen specifically precipitated by the various amounts of capsular polysaccharide both as mg. per cc. of antiserum and in per cent with reference to the maximum amount of specifically precipitable nitrogen. From these data have been calculated the amounts of specific antibody nitrogen which in theory should still be present in the supernatant fluids. That all precipitates (except VII) were formed on the antibody excess side of the equivalence zone was shown by the fact that none of the other supernatant

fluids contained immunologically demonstrable capsular polysaccharide.

The results of the mouse protection tests on the supernatant solutions in terms of dilution end-points (Muench 50 per cent system) are also shown. From these figures by proportional calculation have been derived the number of mouse protective units remaining in the supernatant solutions. The last column gives the number of protective units per mg. of specifically precipitable nitrogen.

TABLE I

Fractional Precipitation of Antipneumococcus Horse Serum with Specific Capsular Polysaccharide and the Determination of the Protective Potency of the Unprecipitated Antibody in the Supernatant Fluid

Supernatant fluids		N₂ specifically precipitated		Supernatant fluids				
				Specific N₂ (by difference)		Mouse protection		
Desig-nation	Obtained after addition of capsular polysaccha-ride mg. per cc. of serum	Mg. per cc.	Per cent of maximum specifically precipitable nitrogen	Mg. per cc.	Per cent of maximum specifically precipitable nitrogen	Dilution end-point	Calculated units per cc. referred to original serum	Protective units per mg. of specific N₂ (protective ratio)
I	0	0	0	1.844	100.0	1–109.4	1405	760
II	0.025	0.559	30.3	1.285	69.7	1–87.7	1125	876
III	0.05	0.923	50.1	0.921	49.9	1–64.0	822	893
IV	0.06	1.124	61.0	0.720	39.0	1–39.7	510	708
V	0.08	1.342	72.8	0.502	27.2	1–13.6	175	349
VI	0.10	1.571	85.3	0.273	14.7	1–4.0	51	187
VII	0.25	1.844	100.0	0	0	—	0	—

From these results it will first be noted that the admixture of 0.25 mg. capsular polysaccharide with 1.0 cc. of serum removed all of the protective antibody. Each addition of polysaccharide removed a certain amount of protective antibody, but the relationship between protective potency and specifically precipitable protein remaining in solution is not linear. Thus it will be noted that with the removal of the first 30 per cent of specifically precipitable nitrogen the antibody remaining in solution showed a higher protective ratio than that in the original unabsorbed serum. The direction of this change, however, was reversed following the removal of 60 per cent of the pre-

cipitable antibody, and as more and more antibody was removed the protective ratio of that remaining in solution became progressively lower, until finally when 84 per cent had been precipitated the antibody remaining possesses a potency of only 187 protective units per mg.

It is obvious that all supernatant fluids previous to VI contained antibody of low protective potency. It is possible, however, to arrive at the actual protective potency of each fraction of precipitable antibody by a process of subtraction. The results of these calculations are shown in Table II and presented diagrammatically in Text-fig. 1.

TABLE II

Analyses of Protective Potencies of Various Fractions of the Specifically Precipitable Antibody of Type I Antipneumococcus Horse Serum

Fraction		Position in precipitable range	Specific nitrogen content of fraction (by difference from Table 1)	Protective units in fraction (by difference from Table 1)	Protective ratios: Units per mg specific N_2
Designation	Reference supernatant designation				
		per cent			
1	I–II	0 –30.3	0.559	280	505
2	II–III	30.3–50.1	0 364	303	830
3	III–IV	50.1–61.0	0.201	312	1550
4	IV–V	61.0–72.8	0 218	335	1540
5	V–VI	72.8–85.3	0 229	124	540
6	VI–VII	85 3–100 0	0 273	51	187

It will be noted that the antibody precipitated by the addition of the smallest amount of capsular polysaccharide possessed a protective ratio (505 units per mg.) much lower than that of the antibody in the unabsorbed serum (760 units per mg.). Similarly the last fraction precipitated (No. 6) possessed an extremely low protective potency (187 units per mg.). Between these two low protective ratios, however, there exists a range in which the mouse protective value is very high, approximately 1550 units per mg. of specifically precipitable nitrogen which is almost exactly twice the figure for the unabsorbed serum.

Repeated experiments as well as estimations of the probable errors

in the work lead to the conclusion that although the actual figures may not be absolute the comparative values are correct.

It is quite apparent from these results that those substances in antipneumococcus horse serum which are considered as specific antibodies do not possess equal properties as regards their capacity to protect mice against infection.
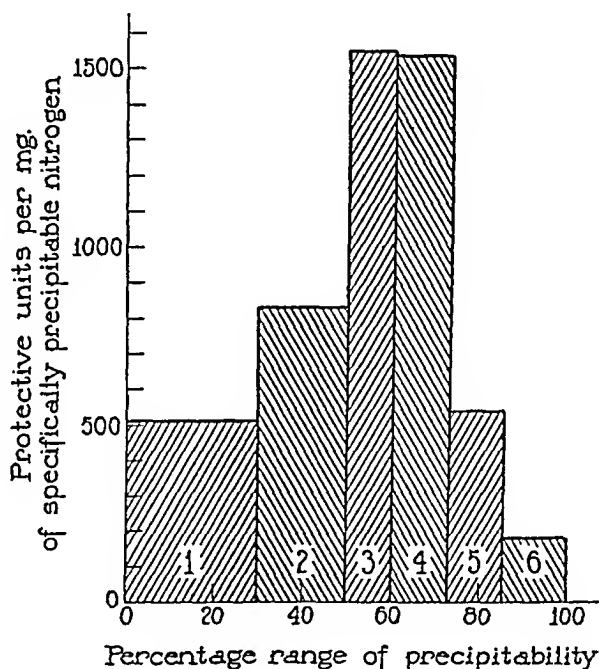
TEXT-FIG. 1. Protective potencies of various fractions of the specifically precipitable antibody of Type I antipneumococcus horse serum.

These results might be interpreted as demonstrating the existence in an immune horse serum of a large series of antibodies of varying potencies. A simpler conception would be that in antipneumococcus horse serum there exist at least three substances possessing all of the properties of specific antibodies. That which is first precipitated upon the addition of polysaccharide might be thought of as possessing a great avidity[1] but for some reason a low protective value. That

[1] The term avidity is here used in the more commonly accepted sense of rate of visible reaction rather than with reference to antigen-antibody union proper.

which occupies the intermediate position has less avidity but a very high mouse protective value. That which is last precipitated has the lowest avidity and also the lowest protective value. The figures obtained in this analysis undoubtedly do not represent the actual values inasmuch as these fractions probably still represent mixtures.

Immunological Fractionation of Antipneumococcus Rabbit Serum

A similar procedure of immunological fractionation has been carried out with Type I antipneumococcus rabbit serum and the results are presented in Table III.

TABLE III

Fractional Precipitation of Antipneumococcus Rabbit Serum with Specific Capsular Polysaccharide and the Determination of the Protective Potency of the Unprecipitated Antibody in the Supernatant Fluid

	Supernatant fluids	N_3 specifically precipitated		Supernatant fluids				
				Specific N_3 (by difference)		Mouse protection		Protective
Designation	Obtained after addition of capsular polysaccharide mg per cc. of serum	Mg. per cc.	Per cent of maximum specifically precipitable nitrogen	Mg. per cc.	Per cent of maximum specifically precipitable nitrogen	Dilution end-point	Calculated units per cc. referred to original serum	units per mg. of specific N_3 (protective ratio)
I	0	0	0	1.533	100 0	1–106.8	1830	1195
II	0 045	0 398	26.0	1.135	74.0	1–78.7	1348	1190
III	0.110	0 936	61.5	0.597	38.5	1–45 1	773	1295
IV	0.150	1 196	78.1	0.337	21.9	1–28.3	485	1440
V	0.350	1.533	100 0	0	0	1–10-8	185	—

The application of this method to immune rabbit serum is rendered somewhat difficult by the fact that the protective antibody is not completely precipitated by the capsular polysaccharide as now prepared. For this reason the apparent number of protective units per mg. of precipitable nitrogen in the supernatant of precipitate VI is infinitely high. This same factor tends to distort somewhat the values in all preceding supernatants. By the determination of agglutinin nitrogen by the method of Heidelberger and Kabat (7) it has been found that at least 6 per cent of the specific antibody is not precipitated by the polysaccharide preparation used. This amount

corresponds quite well with the amount of protective substances remaining in the supernatant solution.

TABLE IV

Analyses of Protective Potencies of Various Fractions of the Specifically Precipitable Antibody of Type I Antipneumococcus Rabbit Serum

Fraction		Position in precipitable range	Specific nitrogen content of fraction (by difference from Table III)	Protective units in fraction (by difference from Table III)	Protective ratios: Units per mg. specific N_2
Designation	Reference supernatant designation				
		per cent			
1	I–II	0 –26.0	0.398	482	1210
2	II–III	26.0–61.5	0.538	575	1170
3	III–IV	61.5–78.1	0.260	288	1105
4	IV–V	78.1–100.0	0.337	300	890
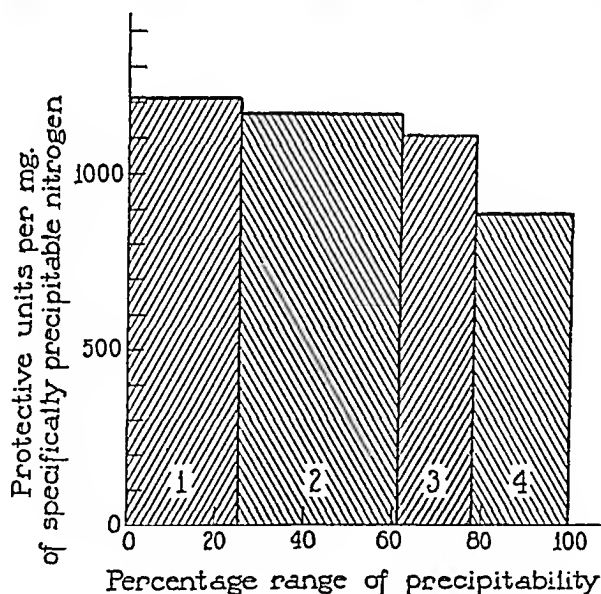
TEXT-FIG. 2. Protective potencies of various fractions of the specifically precipitable antibody of Type I antipneumococcus rabbit serum.

This apparent defect is, however, eliminated by translation of the data, as in the case of horse serum, into terms of protective ratios for

each fraction. These calculations are shown in Table IV and presented graphically in Text-fig. 2.

From these data it will be noted that contrary to the observations with horse serum the antibody first precipitated from rabbit serum has a high protective ratio. In fact this protective value remains high throughout almost the entire range. There is a slight diminution with that antibody which is last precipitated.

The simplest conception which served to describe the results with antipneumococcus rabbit serum is that there are at most only two substances which have all of the properties of specific antibodies and that both possess a relatively high protective ratio. In comparison with antipneumococcus horse serum the differences are slight. These experiments furnish additional evidence as to the marked contrasting properties of the immune sera from the two animal species.

The calculations presented have been based on determinations as to the amount of antibody precipitated, that remaining after precipitation being estimated by difference. In each instance, however, the amount of precipitable antibody in the supernatant fluids was actually determined. Since the determined residual antibody showed a quantitative parallelism with the amount estimated by difference, the calculated protective ratios are so closely parallel to those reported that they need not be detailed.

DISCUSSION AND SUMMARY

The generally held view has been that in any immune serum only a single antibody would be induced by and react with a single antigen. Were this true the various manifestations of antibody activity should show a quantitative parallelism. It has already been shown (1), however, that with antipneumococcus horse serum the mouse protective potency does not parallel the maximum amount of specifically precipitable protein except within certain well defined groups of antisera. The simplest explanation of this situation is that different horses form antibodies differing in specific protective capacity, but from our studies it seems probable that in any immune serum there may occur a mixture of antibodies which, while directed against the same antigen, possess different protective capacities, different avidities, etc. It would now appear that this latter hypothesis is the more tenable since the experiments here reported indicate the existence of antibodies of

various protective potencies in horse antisera.   It would not be unreasonable to hold that the antibodies of a single serum represent a series of substances with varying properties.   On the basis of the present immunological fractionation experiments, the following deductions seem permissible.

1. Antipneumococcus horse sera must contain at least three, possibly many, antibody substances which react with the capsular polysaccharide.   These are: (*a*) A substance which precipitates upon the addition of a relatively small amount of polysaccharide.   This antibody possesses a low protective potency.   (*b*) A substance which is precipitated with intermediate amounts of polysaccharide and which possesses an extremely high protective value.   (*c*) A third substance which is precipitated only with the addition of relatively large amounts of polysaccharide.   The protective value of this antibody is very low It may represent a degraded form.

2. With antipneumococcus rabbit serum the situation is somewhat simpler.   This is in accord with the fact that with Type I antipneumococcus sera from this species there is a direct proportionality between the amount of specifically precipitable protein and the protective potency of the serum (1).   The results with antipneumococcus rabbit serum indicate the existence of at least two antibody substances: (*a*) An antibody with high protective value which makes up the greater proportion of the total content.   (*b*) A second substance which is precipitated only upon the addition of relatively large amounts of capsular polysaccharide.   The existence of this second antibody is not clearly demonstrated by the present findings but the lower protective ratios obtained as greater amounts of antibody are removed probably indicate its existence.   This may also represent degraded material.

The observations on the antibodies of both horse and rabbit antisera will be supported by experiments with immunochemical fractionation which will be reported in a subsequent paper.

CONCLUSIONS

1. In an effort to explain a certain lack of parallelism between the antibody content and the protective values of antipneumococcus horse sera, experiments have been carried out in which determined propor-

tions of the specific antibody have been removed from sera and the protective values of the residuum determined.

2. These experiments with immunological fractionation have revealed that the specific antibodies of immune sera do not possess uniform mouse protective potencies. The inference is that there may be several different substances in immune sera, each dominantly specific, yet exhibiting marked differences in avidity and in protective value.

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PROPERTIES OF THE TYPE SPECIFIC PROTEINS OF ANTIPNEUMOCOCCUS SERA

III. Immunochemical Fractionation of Type I Antipneumococcus Horse and Rabbit Sera

By KENNETH GOODNER, Ph.D., and FRANK L. HORSFALL, Jr., M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

The immediately preceding paper of this series dealt with experiments designed to determine if all type specific antibodies of antipneumococcus serum possessed equivalent mouse protective values (1). The results obtained by comparison of the amounts of antibody protein with the protective potencies of various fractions precipitated by the addition of capsular polysaccharide furnished evidence that in antipneumococcus horse serum there exist at least three, and probably more, substances capable of reacting with the specific carbohydrate. With antipneumococcus rabbit serum evidence of marked differences in the specifically reactive antibodies is not so evident.

Although the results of these experiments were quite definite it seemed desirable to confirm the findings by some independent procedure. The most convincing confirmation would, of course, be the actual isolation of these various antibody substances.

The present paper reports the results of attempts to isolate the specific antibodies of Type I antipneumococcus sera. The method employed was immunochemical rather than purely chemical, since it was obviously important to obtain antibodies specifically directed against the capsular polysaccharide and as free as possible from other protein substances in the serum possessing properties chemically similar, but immunologically unrelated to those which characterize the specific antibody.

EXPERIMENTAL

Materials and Methods.—The general methods used for the determination of specifically precipitable nitrogen and protective potencies have been detailed

in the first paper of this series (2).   The methods employed in the various lipid analyses have also been reported (3).

Immunochemical Fractionation of Type I Antipneumococcus Horse Serum

After several other methods had been tried it was found that the so called dissociation method of Heidelberger and Kendall (4) offered the greatest promise.   This method is selective since it involves recovery of antibodies from the immune precipitate formed by the addition of capsular polysaccharide to the homologous immune serum. The precipitate thus formed is washed and then extracted with hypertonic salt solution.   As a result of this treatment some free antibody appears in the solution.   These precipitates were formed on the antibody excess side of the equivalence zone since in the region of excess polysaccharide the bound antibodies are not freed upon extraction with hypertonic salt solution.

Heidelberger and Kendall using a serum containing both Type I and Type II antibodies found, however, that the immune precipitate formed upon the addition of Type II polysaccharide yielded both Type I and Type II antibodies upon extraction.   According to now prevalent views the Type I antibody could scarcely have been precipitated as a result of true antigen-antibody union, since the addition of Type II polysaccharide to a Type I antiserum does not give a visible precipitate.   Since this experiment was carried out with a polyvalent serum two explanations seemed possible.   First, it might be that an animal immunized to two antigens may form antibodies which in part at least are polyvalent, that is, each antibody molecule might contain groupings which would react with both antigens.   Secondly, it might be that each antibody in polyvalent serum is strictly monovalent but that those of the heterologous type become occluded in the immune precipitate as a result of adsorption.

The possibility of the participation of adsorptive phenomena suggested that the rôle of temperature be investigated.   Consequently an experiment was arranged so that immune precipitates were formed at various temperatures from mixtures of monovalent sera and from a polyvalent serum.

Immune precipitates were prepared from these sera by the addition of Type I pneumococcus capsular polysaccharide at two contrasting temperatures, 4° and

37°C. In each instance the amount of polysaccharide was insufficient to fully saturate the antibody in the serum. After 1 hour the tubes were centrifuged and the precipitates washed twice with saline at the respective temperatures. To each washed precipitate was then added 1.0 cc. of 6.4 per cent NaCl solution and the precipitates were thoroughly dispersed in this fluid. All tubes were incubated 1 hour at 37°C. and were then centrifuged and the supernatant fluid removed and diluted with three volumes of distilled water so as to bring the salt concentration to 1.6 per cent. To portions of these supernatants were then added various polysaccharides in order to detect the presence of homologous and heterologous antibodies.

TABLE I

Effect of Temperature upon the Absolute Specificity of Immune Precipitation

Serum	Poly-saccharide added	Reac-tion	Mixtures incubated 1 hr.; washed twice with saline at respective temperature. 1 cc. 6.4 per cent NaCl added to each tube and all incubated 1 hr. at 37°C. Centrifuged. Supernatant diluted 1–4 with distilled water. Extract tested against following polysaccharides:			
			Type I	Type II	C	Saline
		°C.				
Mixed Type I and Type II........	Type I	37	++++	−		−
Same	" "	4	++++	++		−
Polyvalent Types I and II	" "	37	++++	−	−	−
Same...	" "	4	++++	++	+	−

The results of this experiment are shown in Table I. These data demonstrate that at 4°C. certain amounts of heterologous as well as homologous antibodies had been carried down in the precipitate formed upon the addition of Type I capsular polysaccharide. On the other hand those reactions carried out at 37°C. appear to have been entirely specific since the heterologous antibodies were not extracted from these immune precipitates.

Because of the relationship to temperature it seems reasonable to infer that heterologous antibodies may become involved in the formation of immune precipitates as a result of adsorption. Furthermore these results suggest that antibodies are relatively monovalent even though formed at the same time as a result of two specific stimuli.

With the knowledge that only the specific antibodies are precipi-

Protocol of Experiment for the Selective Isolation of t

Type I antipneumococcus horse serum lot 1150. 200 cc. d

Precipitate.—Washed twice with tap water. Added
until dissolved. Warmed to 37°C. and at this t
SSS added. Precipitate allowed to settle 1 hr. 37

Precipitate.—Washed with physiological saline 3 times 37°, 100 c
added, agitated 1 hr. 37°. Centrifuged

Precipitate.—Discarded

Supernatant.—Filtered through paper.
Filtrate dialyzed 48 hrs. against
running tap water. Centrifuged

Precipitate.—Added 100 cc.
1.6% NaCl—thorough agi-
tation. Centrifuged

Supernatant.—
with capsul
Active mate
pH to 7.65.
sin presence
able. Desig

Insoluble fraction.—After treatment
with trypsin gives reaction with
antiserum indicating presence of
combined polysaccharide

Soluble fraction.—Gives immediate reaction
with capsular polysaccharide. Precipi-
tates upon dilution with water. After
digestion with trypsin presence of poly-
saccharide not detectable. Designated
as E antibody

II

Protocol of Experiment [in which the] Specific Antibodies of Antipneumococcus Horse Serum

[...] antipneumococcus horse serum [...] plus 1800 cc. tap water—overnight in ice box. Centrifuged

Precipitate.—Washed [...] 1.6% NaCl; stirred
[...] dissolved Warmed to temperature 10 mg. Type I
SSS added Precipitate dissolved centrifuged 1 hr. 37°   *Supernatant.*—Discarded

—Washed with physiological saline 1.6% NaCl
[...] 1 hr 37° Centrifuged   *Supernatant.*—10 mg. SSS I added 37°.
                                                After 1 hr. 37°, centrifuged

         Supernatant.—[...]   *Precipitate.*—Washed with physiological saline   *Supernatant.*—Discarded
         Filtrate dialyzed [...]   3 times 37°. 100 cc. 10% NaCl added; agi-
         running tap water [...]   tated 1 hr. 37°. Centrifuged

Added 100 cc.
[...]   Immediate precipitation   *Precipitate.*—Discarded   *Supernatant.*—Filtered through paper.
[...]   polysaccharide in saline.   Filtrate dialyzed 48 hrs. against
[...]   precipitated on adjusting   running tap water. Centrifuged
                     [...] digestion with tryp-
                     polysaccharide not detect-
                     [...] as P antibody

fraction.—Gives immediate reaction   *Precipitate.*—Added 100 cc.   *Supernatant.*—Discarded—Gives
capsular polysaccharide. Preci-   1.6% NaCl—thorough agi-   no reaction with SSS
upon dilution with water. After   tation. Centrifuged
[...] with trypsin presence of poly-
[...] not detectable. Desigated
antibody
                                                  Insoluble.—Discarded   *Soluble fraction.*—Properties as E

tated at 37°C. and that release of antibody can only be obtained on the antibody excess side of the equivalence zone, a method was available for the selective isolation of capsular carbohydrate antibodies from antipneumococcus sera.

The procedure adopted with antipneumococcus horse serum can best be presented in the form of a protocol as in Table II. The serum is first diluted with water in order to precipitate the so called water-insoluble globulins. The water-insoluble fraction contains most of the type specific antibodies and by this initial procedure a certain degree of purification is effected by elimination of a considerable amount of inert material. The protein precipitate is then dissolved in 1.6 per cent NaCl and warmed to 37°C. At this temperature a small amount of the capsular polysaccharide is added and the resulting precipitate allowed to settle for 1 hour. After this time the supernatant fluid is removed and the precipitate washed three times with warmed saline by centrifugation. To the precipitate is then added an appropriate amount of 10 per cent NaCl and the contents are agitated for 1 hour. The supernatant fluid is then filtered through paper and dialyzed 48 hours against running tap water. In the meantime to the supernatant resulting after the first immune precipitation an additional amount of specific polysaccharide was added and the resulting precipitate extracted in exactly the same manner. Addition of small amounts of polysaccharide have been used rather than one larger amount so as to obtain precipitates representing different points in the scale of avidity.

From the hypertonic salt extracts of these immune precipitates, as well as those of all experiments, three separate fractions were recovered. The first fraction, usually present in all extracts, consists of material which precipitated during dialysis and was not then soluble in 1.6 per cent salt solution and was only sparingly soluble in more concentrated salt solutions. No evidence was found of the presence of free polysaccharide in this material, although after heating to denature the protein and treating the coagulum for a few minutes with trypsin at pH 8.2 a strong reaction was obtained with immune serum indicating that the enzymatic digestion had released free polysaccharide. This fraction is regarded as a combined polysaccharide-antibody complex.

A second fraction, protein in nature, which also precipitated as a result of dialysis was completely soluble in 1.6 per cent salt solution. The resulting viscous opalescent solution gave a strong reaction with capsular polysaccharide and as will be shown below had excellent

mouse protective properties. This substance is designated as antibody E.

The hypertonic salt solution extract of the precipitate obtained upon the first addition of the polysaccharide to the antibody solution contained a third material which did not precipitate upon dialysis but which nevertheless gave strong reactions with added polysaccharide. This protein was water-soluble except in the neighborhood of pH 7.65. If brought to this point the protein associated with antibody activity separated out slowly as a fine white precipitate. Solutions of this material also possessed the property of protecting mice. This form of antibody, designated as P, is usually obtained only from the first precipitate formed on the addition of the polysaccharide.

This method of fractionation has been repeatedly carried out with various lots of immune horse sera and has invariably yielded these two forms of type specific antibody. The various determined properties of the two antibody solutions may be summarized as follows:

Solubilities.—The E antibody is water-insoluble and is precipitated from solution by third saturation with ammonium sulfate. In this respect it resembles an euglobulin. The P antibody is water-soluble except at its isoelectric point, approximately pH 7.6. It is not precipitated until half saturation with ammonium sulfate is reached. It thus resembles a pseudoglobulin and in these properties it appears to be identical with the material described by Goebel and Chow (5).

Avidities.—If solutions of these two antibodies are diluted to the same protein concentration and similar amounts of capsular polysaccharide added, care being taken to avoid convection currents, it is found that the P antibody precipitates much more rapidly. In this respect it may be thought of as possessing the greater avidity, the latter term having been generally used to indicate rate of precipitation rather than rate of union. This finding is supported by the fact of isolation of the P form from the precipitate formed on the addition to the serum of the smallest amount of polysaccharide.

Combining Ratios.—In the range of antibody excess the following mean combining ratios have been determined.

> E: 2.6 mg. nitrogen per mg. capsular polysaccharide
> P: 23.4 "   "   "   "   "   "

These combining ratios are consistent with the observations as to the respective avidities of the two forms of antibody.

Heterologous Reactivity.—Type I antipneumococcus horse serum gives few heterologous cross reactions. Goebel and Hotchkiss (6) have shown, however,

that if galacturonic acid (a constituent of the capsular polysaccharide) is attached to a protein, the resultant conjugated antigen gives a precipitin reaction when added to Type I horse serum.   This reaction is not obtained with Type I antipneumococcus rabbit serum nor is it possible with this antigen to remove all of the type specific antibody from the immune horse serum.   It has been found that this antigen gives a precipitin reaction with solutions of the P antibody but does not react with the E antibody.

Specific Precipitability.—As a means of determining the relative state of immunological purity of the two forms of antibody isolated by the methods described, analyses have been carried out to determine the specific precipitability of various preparations.   Results of analyses on two separate preparations of the E and P antibodies are shown in Table III.

TABLE III

Specific Precipitability of Antibody Preparations

Form	E antibody		P antibody	
Preparation No.	*1*	*2*	*1*	*2*
Total nitrogen, *mg. per cc.*	0.154	0.183	0.141	0.445
Amount of capsular polysaccharide required for maximum precipitation, *mg. per cc.*	0.20	0.10	0.10	0.15
Total nitrogen in precipitate from 1.0 cc. of antibody preparation, *mg.*	0.161	0.187	0.130	0.408
Less correction for nitrogen of polysaccharide in immune precipitate (estimated on basis of 75 per cent of that added), *mg.*	0.008	0.004	0.004	0.006
Net protein nitrogen in precipitate, mg. per cc.	0.153	0.183	0.126	0.402
Specific precipitability, *per cent*	99.4	100.0	89.3	90.3

It will be noted that the determined figures have been corrected for the amount of polysaccharide nitrogen probably present in the precipitate.   It is assumed on the basis of results with whole serum that approximately 75 per cent of the added polysaccharide is combined in the immune precipitate in the range of maximum precipitation.   The figures show that preparations of the E antibody have been obtained in a state of complete precipitability with the specific polysaccharide, that is, immunological purity.   With the P antibody, on the other hand, no preparation has been obtained which is completely precipitable upon the addition of the specific polysaccharide.   It is not as yet certain whether these latter preparations should be regarded as impure or whether a solubility coefficient must be considered.   In view of the more soluble nature of the P antibody itself it would not be surprising if the antigen-antibody complex formed from it were also more soluble.

Lipid Content.—Analyses for lipid carbon and lipid phosphorus, carried out

by the method of Kirk, Page, and Van Slyke (7), gave the following information.

Antibody	Total lipid, mg. per mg. of protein	Phosphatide, per cent of total lipid
E	0.044	22.5
P	0.034	12.5

If the antibody is regarded as a protein-lipid complex containing the determined amount of lipid, one obtains the following per cent content. E, 4.22 per cent; P, 3.29 per cent. It is of interest to note that these figures are in close agreement with those obtained in previous studies on the relation of the phospholipids to the reactivity of immune sera (8).

If one assumes that the antibody proteins are built up of units of molecular weight approximating 34,500 it is easily calculated that in the E antibody there

TABLE IV

Mouse Protective Potencies of Antibody Preparations

	Antibody E Preparation 2	Antibody P Preparation 1
Dilution end-point...................................	1–21.2	1–2.9
Mouse protective units per cc. (simultaneous titration of standard (1000 units per cc.) = 1–75).............	283	39
Maximum specifically precipitable nitrogen (corrected for polysaccharide nitrogen) *mg. per cc.*...................	0.183	0.126
Mouse protective units per mg. of specifically precipitable nitrogen..	1550	230

is one phosphatide molecule (m.w. 778) for every two protein units. With the P antibody the result is one molecule of phosphatide per 5 protein units. In order to determine if the phosphatide were lecithin or cephalin, the antibody preparations were analyzed for the presence of lipid-amino-nitrogen. None was detectable in either the P or the E antibody solutions. The inference is therefore that the phosphatide in both instances is lecithin.

It cannot be absolutely stated that the specific antibodies in immune horse serum are lipoproteins although the present results as well as those previously obtained strongly support this view. Furthermore the finding that lecithin is closely associated with both the P and the E antibody confirms the results of previous qualitative tests in which it was found that the *in vitro* reactivity of lipid-extracted antipneumococcus horse serum could be restored by the addition of very small amounts of lecithin (8). This additional evidence lends further support to the view that the type specific antibodies of antipneumococcus horse serum are lecithoproteins.

Mouse Protective Potencies.—Mouse protective titrations have been carried out by the method outlined in a previous paper (2), one set of data so obtained for each antibody preparation is summarized in Table IV.

The results show marked differences in the mouse protective potencies of the two antibodies.   The more avid antibody, P, possessed only 230 mouse protective units per mg. of specifically precipitable nitrogen, while E gave results indicating 1550 units per mg.

By reference to Table II and Text-fig. 1 of the previous paper (Paper II of this series (1)) it will be noted that the first antibody precipitated on immunological fractionation contained approximately 500 protective units per mg. of specifically precipitable nitrogen.   In view of the present findings this fraction may be regarded as a mixture of the P and the E antibody although consisting largely of the former.   The fractions designated as 3 and 4 in the preceding paper are considered to represent almost pure E antibody since the protective ratio of these fractions was practically identical with that of the isolated form.

Immunochemical Fractionation of Antipneumococcus Rabbit Serum

Somewhat similar procedures have also been applied to antipneumococcus rabbit serum but the work has not progressed to the point which warrants definitive conclusions.

Since the specific antibodies of antipneumococcus rabbit serum are not precipitated when the serum is diluted with water, it has not been possible to eliminate much of the inert material by this initial step. It has been necessary, therefore, to obtain the immune precipitates with the serum directly and in order to approach purity it has been necessary to repeat this procedure with antibody solutions.   Consequently the recovery of antibody from rabbit serum has been low and the lack of material has precluded a systematic analysis.

However, on the basis of the experimental data available certain tentative statements may be made.

It has not been possible to recover from immune rabbit serum a water-soluble antibody corresponding to the so called P antibody of immune horse serum. The active material recovered has been water-insoluble, thus corresponding to the E antibody of horse serum. No claim for immunological purity of this substance can be made on the basis of the few analyses performed but it appears to be at least 85 per cent precipitable by the specific capsular polysaccharide. The mouse protective potency of the E antibody of immune rabbit serum is high, approximately 1100 units per mg. of specifically precipitable nitrogen.

These tentative findings are in complete accord with the results of immunological fractionation experiments presented in the previous paper of this series (1).

SUMMARY AND DISCUSSION

The immunological fractionation of Type I antipneumococcus horse serum, reported in the preceding paper of this series, indicated the existence of at least three forms of type specific antibody (1). This is the simplest interpretation of the results although it is possible that there exists a long series of closely related forms of antibody. Immunochemical fractionation, as reported in the present paper, indicates the existence of at least two distinct forms, the third not being demonstrable by the method employed since it is precipitated only with excess antigen. The three postulated forms of antibody of antipneumococcus horse serum together with certain of their properties may be briefly summarized as follows:

1. The So Called P Antibody.—Water-soluble (except at isoelectric point pH 7.6), pseudoglobulin-like substance (probably a lecithoprotein) which is precipitated from solution by the addition of small amounts of specific polysaccharide. Its specificity is not absolute and its capacity to protect mice is low (230 units per mg. of specifically precipitable nitrogen).

2. The So Called E Antibody.—Water-insoluble, euglobulin-like substance (probably a lecithoprotein) with low specific combining ratio. This antibody has been isolated in a state of immunological purity. Its capacity to protect mice is very high.

3. A Third Antibody Form.—Chemical properties are unknown since it has not been isolated. From immunological studies its existence may be postulated. Relatively large amounts of specific polysaccharide are required for its precipitation and the present evidence indicates a very low mouse protective capacity.

With antipneumococcus rabbit serum the evidence points to the existence of not more than two antibody forms. The first comprises a large proportion of the total specific antibody and in most of its properties it corresponds to the E antibody of immune horse serum. It has a high mouse protective value. The existence of a second form of antibody (corresponding to the third of horse serum) was not conclusively demonstrated by immunological experiments, but the fact that the last fraction of antibody precipitated from rabbit serum upon the addition of the specific polysaccharide had a somewhat lower protective value may indicate that in the rabbit serum as in that from the horse there is a final form, possibly representing degraded material, with lower protective value. These experiments offer evidence that

the injection of a complex antigen, the Pneumococcus, gives rise in certain animals to more than one form of antibody reactive with the type specific capsular polysaccharide.   No explanation for this fact is now available.

It is possible that in immune serum some form of equilibrium may exist between the various antibody forms, this equilibrium being conditioned by secondary components, as for example lipids.   This last thesis would require that the two major antibody forms in antipneumococcus horse sera should be interconvertable, that is, it should be possible to derive the P form from the E.   As yet there is no experimental justification for this point of view.

CONCLUSIONS

Immunological and immunochemical fractionation of Type I antipneumococcus horse and rabbit sera have demonstrated the existence of several forms of immune protein, each fraction behaving as a type specific antibody, but differing from the others in chemical and immunological properties.

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THE ABSORPTION OF PROTEIN SOLUTIONS FROM THE PULMONARY ALVEOLI

By CECIL K. DRINKER, M.D., MADELEINE FIELD WARREN, PH.D., AND MARGARET MacLANAHAN

(From the Department of Physiology, The Harvard School of Public Health, and the Department of Bacteriology, The Harvard Medical School, Boston)

In a recent paper on the permeability of the lungs to antibodies, Fox (1936) has summarized the literature upon the subject and makes it clear both from his own experiments and from those of others that antibodies of "different types contained in foreign or homologous serum appear in the blood stream but slowly after intratracheal injection" and that sterile inflammation of the lungs did not affect this result. Fox made no effort to identify the possible routes of absorption whether directly into the blood or *via* the lymphatics, though he assumes the blood route was the one selected and our experiments show this to be correct. His procedure consisted in intratracheal injections of immune sera in rabbits with sampling of the blood beginning shortly after the injection and continuing for as long as 5 days.

The experiments described below had as their objectives the determination of the route, whether lymphatic or vascular, by which passage of various species of proteins from the pulmonary alveoli was effected, and the rapidity with which these substances which varied in molecular size emigrated from the pulmonary site to the blood and lymph systems. As experimental animals dogs were selected since in them it is readily possible to tie off the lymphatic entrances into the right subclavian vein. If then the thoracic duct is cannulated the lymph drainage of the lungs has been excluded from entrance into the blood, and if samples of thoracic duct lymph and blood are titrated for the presence of foreign protein one can discover the pathway of absorption, whether it be across the alveolar wall and into the blood capillaries or by the lymphatic route to the root of the lung and the thoracic duct.

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Materials and Methods

Dogs anesthetized by intravenous or intraperitoneal injections of barbital-sodium, 0.35 gm. per kilo, were used in all experiments. Animals so anesthetized will often live for several days if given fluid and kept warm, and in most of them blood pressure and general conditions are excellent for 30 hours, a period quite long enough to satisfy the needs of the experiments to be described. In preparation for the experiment the thoracic duct was isolated and tied, care being taken to search for branches making separate entrance into the subclavian vein, since, if lymphatic absorption occurred, such branches would cause contamination of the blood. Through a second incision all lymphatics entering the right subclavian vein were ligatured. The situation so produced probably results in diverting the lymph drainage of the right lung to the thoracic duct, since Baum (1918) has shown that in the dog the lymphatics from the two sides communicate quite freely as they reach the tracheobronchial lymph nodes. In any event the arrangement will provide all the lymph leaving the left lung, and in all experiments effort was made to deposit the solutions used upon that side. While in most cases the left side was shown at autopsy to contain far the larger part of the injected material, some of the solution invariably reached the right lung.

Following the two operations upon the lymphatics the trachea was cannulated and in some instances preparations made to take blood pressure, and in one case to perform plasmapheresis. To introduce the solutions into the lungs, the board was elevated at the head end and a long glass catheter with a slight bend at the end to assist entrance into the left bronchus was inserted slowly and without disturbance to the animal. The amounts of solution injected varied in different animals from 20 to 50 cc. Usually they were run in at a single injection, the rate being slowed if respiration became active, and the elevated position maintained for about an hour. The animals were killed by bleeding and an autopsy performed at once. Sections of the parts of the lungs containing solutions as well as from the tracheobronchial lymph nodes were made for microscopic examination.

Precipitating sera were obtained from rabbits injected intravenously with normal horse serum, crystallized egg white, and crystallized horse hemoglobin. Horse serum, obtained from a single bleeding of one animal, was used for preparation of the antiserum and for the intratracheal injections. This anti-horse-rabbit serum exhibited a titre of 1:10,000 to 1:14,000. The egg white and hemoglobin were crystallized, the first by the method of Sørensen (1917) and the second by the method of Green (1931). The egg albumin antiserum had a titre of 1:30,000 and the crystallized hemoglobin of 1:500. Serum and lymph titrations were begun at one-half dilution and continued through various dilutions. The precipitin tests were carried out by means of the ring technique using undiluted antiserum. Readings were taken at the end of 1 hour's incubation at room temperature. Physiological salt solution was used to dilute blood serum and lymph under examination for egg white and hemoglobin antibodies, but 1.5 per

cent salt solution proved more satisfactory in the case of these fluids derived from the dogs receiving intrabronchial administration of horse serum. Controls included the titration of the dog blood and lymph before injection as well as the usual saline control.

EXPERIMENTS

Experiment 1. Intratracheal Horse Serum.—Jan. 28, 1937. Dog weighing 7.7 kilos. 10:15 a.m. 25 cc. of 10 per cent barbital-sodium solution intravenously. 11:45. Thoracic duct cannulated. Lymphatic entrances on right ligatured. Trachea cannulated.

11:50. Lymph specimen 1 (control).

12:00. Blood specimen 1 (control). 200 cc. physiological saline intravenously. 12:10 p.m. *15 cc. of horse serum plus 2.5 cc. graphite suspension injected*

TABLE I

Titration of Blood Samples with Anti-Horse-Rabbit Serum.   Dog 1

Time	Speci-men	Antiserum dilutions			Saline control	Time after intratracheal injection of horse serum
		1:3	1:6	1:12		
12:00 noon..............	1	0	0	0	0	Control specimen
4:15 p.m................	2	0	0	0	0	4 hrs. and 5 min.
10:45 p.m...............	3	0	0	0	0	10 hrs. and 35 min.
8:40 a.m....	4	++	+	Trace	0	20 hrs. and 30 min.
12:15 p.m...............	5	++	+	Trace	0	24 hrs. and 5 min.

intratracheally. 12:15. Rectal temperature 100.1°F. 12:25. *15 cc. of horse serum plus 2.5 cc. graphite suspension injected intratracheally.*

4:15. Rectal temperature 97.2°F. Lymph specimen 2. Blood specimen 2. 4:35. 60 cc. 20 per cent glucose solution plus 180 cc. of water by stomach tube.

10:45. Lymph specimen 3. Blood specimen 3. 10:50. Rectal temperature 96.6°F. 50 cc. water by stomach tube.

Jan. 29. 8:40 a.m. Lymph specimen 4. Blood specimen 4. 9:05. Rectal temperature 96.3°F. 9:10. 100 cc. physiological saline intravenously. Lymph flowing well. 12:05 p.m. Rectal temperature 95.2°F.

12:15. Lymph specimen 5. Blood specimen 5. 12:20. Bled to death. Blood flowed well indicating a fairly high blood pressure. Duration of experiment from the time of the first injection of horse serum 24 hours and 10 minutes.

*Autopsy.—*Practically all of the graphite colored injection was in the *right* lower lobe, only a trace having reached the left lung. Microscopic sections of blocks of the right lung taken from serum-containing areas showed much pink stained serum in the alveoli and many particles of graphite both free and in phago-

cytes. There was no perceptible accumulation of graphite in lymphatics. Tracheobronchial lymph nodes showed no accumulation of serum or graphite in the sinuses.

Table I shows the results of serological examination of the blood. Similar examinations of lymph were all negative.

Summary of Experiment 1.—In this case only a small fraction of the injected serum reached the left lung, which is in the main line of lymph drainage in the preparation employed. After 10 hours and 35 minutes no horse protein could be detected in the blood. There followed an interval of 9 hours and 55 minutes, and then the foreign protein was found in the blood. Lymph was consistently negative. The conclusion appears unescapable that the horse protein passed through the pulmonary epithelium and directly into the blood capillaries.

Experiment 2. Intratracheal Horse Serum.—Jan. 19, 1937. Dog weighing 13.5 kilos. Anesthesia and preparations as in Experiment 1. 3:30–3:45 p.m. *30 cc. horse serum plus a small amount of trypan blue intratracheally.*

Jan. 20. 10:00 a.m. Animal bled to death.

Autopsy.—The serum and trypan blue were distributed in both lower lobes, which were dark red and solid. The left side on cross-section contained somewhat more. Microscopical examination showed much intra-alveolar serum with many polymorphonuclear leucocytes scattered through it. The tracheobronchial lymph node sections were normal.

Tables II and III show the results of serological examination.

Summary of Experiment 2.—In dog 2 a large part of the injected serum reached the left lung. Horse protein was found in blood and lymph 17 hours and 5 minutes after intratracheal injection, but the concentration was greater in the blood. This result was obtained on other occasions, and in our opinion expresses the fact that after foreign protein accumulates in the blood one may expect transfer of it to the thoracic duct lymph in the abdominal region, where both in the liver and intestine capillary permeability to protein is high. The rise in concentration in the lymph parallels that in the blood and is a further indication of natural transfer.

Experiment 3. Intratracheal Horse Serum Followed by Plasmapheresis.—Mar. 9, 1937. Dog weighing 13 kilos. Anesthesia and general preparations as in Experiment 1, with the addition of cannulation of the femoral artery and vein in preparation for plasmapheresis.

10:50 a.m. Plasmapheresis started and carried on until 3:15 p.m. when the blood protein was reduced from 7.6 per cent to 4.2 per cent. Under these circumstances thoracic duct lymph flows very freely. 3:35 p.m. 35 cc. of horse serum were given intratracheally. This serum contained 6.6 per cent protein. Specimens of lymph and blood were taken for 3 hours and 10 minutes. None contained detectable amounts of horse protein.

TABLE II

Titration of Blood Samples with Anti-Horse-Rabbit Serum.   Dog 2

Time	Specimen	Antiserum dilutions					Saline control	Time after intratracheal injection of horse serum
		1:2	1:4	1:8	1:16	1:32		
3:15 p.m.	1	0	0	0	0		0	Control specimen
10:00 p m.	2	0	0	0	0			6 hrs. and 30 min.
8:35 a.m.	3	+	+	0	0			17 hrs. and 5 min.
12:00 noon	4	+	+	+	0			20 hrs. and 30 min.
3:45 p.m.	5	++	+	+	0	0		24 hrs. and 15 min.
9:50 p.m.	6	++	++	+		0		30 hrs. and 10 min.

TABLE III

Titration of Lymph Samples with Anti-Horse-Rabbit Serum.   Dog 2

Time	Specimen	Antiserum dilutions					Saline control	Time after intratracheal injection of horse serum
		0	1:2	1:3	1:6	1:12		
3:10 p.m.	1	0	0	0	0	0	0	Control specimen
10:00 p.m.	2	0	0	0	0	0	0	6 hrs. and 30 min.
8:35 a.m.	3	+	Trace?	0	0	0	0	17 hrs. and 5 min.
12:00 noon.	4	++	+	+	0	0		20 hrs. and 30 min.
3:45 p.m.	5	++	++	+	0	0		24 hrs. and 15 min.
9:50 p.m.	6	++	++	+	+	0		30 hrs. and 10 min.

Summary of Experiment 3.—In this case neither the rapid flow of lymph nor the fact that the intra-alveolar protein had a higher concentration than the blood protein of the dog had any marked effect on absorption from the alveoli. Had the experiment continued longer some acceleration might have been observed, but since at the close of the experiment the blood protein had risen to 6.5 per cent it is improbable that the result would have differed greatly from that observed in Experiments 1 and 2.

Experiment 4.   Intratracheal Horse Serum in a Dog Sensitized to Horse Serum.— *Jan. 25, 1937.*   Dog weighing 21 kilos.   50 cc. horse serum intravenously.

Mar. 3.   10 cc. horse serum intravenously.

Mar. 27.   Blood shows no horse protein.

Mar. 30.   9:30 a.m.   Anesthesia and preparations as in Experiment 1 plus cannulation of femoral artery for blood pressure measurements.   11:45.   After taking control specimens, *30 cc. of horse serum were given intratracheally.*   There was no increase in lymph flow, and blood pressure tracings taken during the next 9 hours and 45 minutes showed no disturbance of any sort.   9:30 p.m.   *10 cc. of horse serum were given intravenously* to find out whether the animal was sensitized. This injection produced a slight fall in blood pressure and an enormous increase in lymph flow,—typical effect of anaphylactic shock in the dog (Petersen and Hughes, 1925).

During this entire experiment no horse protein was found in blood or lymph.   The animal was evidently sensitized but did not absorb enough horse protein from the alveolar reservoir to cause shock.

Experiment 5.   Intratracheal Horse Serum with a Forced Increase in Breathing.— *Apr. 20, 1937.*   Dog weighing 11 kilos.   In this case all preparations were as in Experiment 1 except that the animal was forced to rebreathe, thus greatly accelerating and deepening its respiration.   Specimens of blood and lymph were taken during 5 hours and 25 minutes.   No horse serum was detected in either fluid, and it is evident that even the most vigorous breathing has no effect on the absorption of horse protein.

Experiment 6.   Intratracheal Crystallized Hemoglobin.—Apr. 15, 1937.   Dog weighing 14.5 kilos.   Anesthesia and preparations as in Experiment 1.   10:35 a.m.   *25 cc. of 20 per cent hemoglobin solution injected intratracheally.*

Apr. 16.   10:45 a.m.   The dog was bled to death.   At autopsy the injected hemoglobin was about equally divided between the right and left lower lobes. Microscopically the hemoglobin-containing portions of the lung show a large number of polymorphonuclear leucocytes.   No concentration of hemoglobin in lymphatics or in the sinuses of the lymph nodes from the root of the lung can be detected.

Table IV shows the results of serological examination of the blood.   13 hours and 28 minutes passed before hemoglobin was detected with certainty in the blood. Lymph titrated in a similar manner throughout the experiment never was shown to contain hemoglobin.

Summary of Experiment 6.—The absorption of hemoglobin is directly into the blood vessels and seems to be entirely similar to the absorption of horse serum protein.

Experiment 7.   Intratracheal Crystallized Egg Albumin in Physiological Saline.— *Mar. 15, 1937.*   Dog weighing 17 kilos.   Anesthesia and preparations as in Experi-

TABLE IV

Titration of Blood Samples with Anti-Hemoglobin-Rabbit Serum

Time	Speci-men	Antiserum dilutions			Saline control	Time after intratracheal injection of hemoglobin
		1:2	1:4	1:6		
10:05 a.m............	1	0	0	0	0	Control specimen
12:03 p.m......... ...	2	0	0	0	0	1 hr. and 28 min.
2:10 p.m............	3	0	Trace	0	0	3 hrs. and 35 min.
4:00 p.m............	4	0	Trace	0	0	5 hrs. and 25 min.
5:00 p.m............	5	Trace	0	0	0	6 hrs. and 25 min.
8:00 p.m............	6	0	0	Trace	0	9 hrs. and 25 min
12:03 a.m...........	7	+	0	Trace	0	13 hrs. and 28 min.
8:30 a.m......	8	+	+	0	0	21 hrs. and 55 min.
10:45 a.m...........	9	+	+	+	0	24 hrs. and 10 min.

TABLE V

Titration of Blood Samples with Anti-Egg-Albumin-Rabbit Serum

Time	Speci-men	Antiserum dilutions				Saline control	Time after intratracheal injection of egg albumin
		1:2	1:4	1:6	1:8		
11:35 a.m..............	1	+	0	0	0	0	Control specimen
1:45 p.m..............	2	+	+	+	0	0	2 hrs.
3:45 p.m..............	3	+++	++	++	+	0	4 hrs.
4:55 p.m..............	4	++	++	+	++	0	6 hrs. and 10 min.
7:55 p.m...	5	++	++	+	0	0	9 hrs. and 10 min.

TABLE VI

Titration of Lymph Samples with Anti-Egg-Albumin-Rabbit Serum

Time	Speci-men	Antiserum dilutions				Saline control	Time after intratracheal injection of egg albumin
		1:2	1:4	1:6	1:8		
11:35 a.m..............	1	0	0	0	0	0	Control specimen
1:45 p.m..............	2	0	0	0	0	0	2 hrs.
3:45 p.m.............	3	+++	++	++	+	0	4 hrs.
4:55 p.m.	4	+++	+++	++	++	0	6 hrs. and 10 min.
7:55 p.m..............	5	+++	+++	++	++	0	9 hrs. and 10 min.

ment 1. 11:45. 39 cc. egg albumin solution given intratracheally. This solution contained approximately 6 per cent of egg albumin. No evidence of irritation.

Mar. 16. Dog was found dead in the morning, the tracheal cannula being plugged with mucus and froth.

Tables V and VI show the results of serological examinations.

Summary of Experiment 7.—In this case absorption into the blood was prompt and appearance in the lymph soon follows. The somewhat higher concentration of egg albumin in the lymph found late in the experiment occurred in another similar experiment and perhaps indicates some degree of absorption by the lung lymphatics, but in any event this was slight.

<div align="center">DISCUSSION</div>

The results of these experiments were not at all in accord with our expectations. It is known that physiological salt solution is absorbed rapidly from the pulmonary alveoli and undoubtedly passes directly into the blood (Winternitz and Smith, 1920). In contrast to this direct route, particles of various sorts which reach the alveoli find their way into the lymphatics and are removed by the lymphatic route. It is generally thought that intra-alveolar phagocytosis is a necessary first step in removal of particulate material from the alveoli, but many experiments have shown that particles so deposited reach the lymph nodes at the root of the lung far too rapidly to permit phagocytosis as a first step (Drinker and Field, 1933). It has also been shown that respiratory movements have an important influence upon particle absorption, it being extremely slow in a motionless lung (Shingu, 1908). By analogy with the fact that protein solutions injected subcutaneously are removed practically entirely by the lymphatics and that this is true for such solutions when deposited in the serous cavities and in practically all parts of the body, it had been our belief that intra-alveolar protein would depart by the lymphatic route and in view of the results with visible particles we were prepared to find this absorption rather rapid and accelerated by deep breathing. Neither of these expectations was realized. Absorption was exceedingly slow and of very small volume. It was directly into the blood vessels and the rate and volume of absorption were not affected by increase in breathing, by sensitization, or by reducing the concentration of the blood proteins by plasmapheresis. Horse serum and horse hemoglobin were absorbed very slowly and at about an equal rate.

A great deal is known about the anatomy and histology of the lung lymphatics, but the reasons for their very extensive distribution are by no means clear. Owing to the fact that the blood pressure

in the pulmonary capillaries is probably much below the colloid osmotic pressure of the blood, a situation exists very favorable to the absorption of water by the blood capillaries, and this in turn is a favorable arrangement in an organ where gas transfer is preeminently important. Elsewhere in the body an important function of the lymphatics is to carry away blood proteins which have passed through the walls of the capillaries to become constituents of the tissue fluid, but in the lungs the situation does not favor tissue fluid production, and one is forced to believe that the very elaborate lymphatic system described by Miller (1937) exists to function in the presence of infection, hemorrhage, foreign body deposition, in short against any process which results in the presence of foreign material in the lung tissue. In order to enter lung lymphatics it is essential to traverse a certain amount of lung tissue since lymphatic capillaries do not extend past the alveolar ducts. Apparently particles of inorganic material and bacteria make this transit in the breathing lung, but fluids actually in the alveoli and appropriate to removal by the lymphatic route do not succeed in it. Even very vigorous breathing, which greatly accelerates the movement of intra-alveolar particles into the lymphatics, fails to bring about lymphatic absorption of fluids.

SUMMARY

Horse serum, crystallized hemoglobin, and crystallized egg albumin have been injected into the lung alveoli of dogs in which the entrances of the right lymphatics have been tied and the thoracic duct cannulated. Samples of blood and lymph have been taken following this injection. Only after several hours in the case of the horse serum and hemoglobin have these proteins been detected by immunological methods and invariably they have appeared first in the blood. Egg albumin also enters the blood capillaries, but much more rapidly than the other two proteins, due probably to the smaller molecular size.

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FURTHER OBSERVATIONS ON VITAMIN C THERAPY IN EXPERIMENTAL POLIOMYELITIS*

By CLAUS W. JUNGEBLUT, M.D.

(From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York)

We have previously presented data to show that the parenteral administration of natural vitamin C during the incubation period of poliomyelitis in experimentally infected monkeys is followed by a distinct change in the severity of the disease (1). This was indicated by the fact that among a total of 62 C-treated animals 19 survived without paralysis and 43 succumbed to the disease, while of a total of 38 untreated control animals only 2 failed to develop paralysis and 36 succumbed to the disease. It appeared therefore that about 30 per cent of all monkeys which had received injections of ascorbic acid escaped paralysis as contrasted with approximately 5 per cent of non-paralytic survivors among the controls, a margin sufficiently large to admit of little possibility that the observed difference in severity of the disease could have been accidental. However, in view of the fact that the virus failed to produce paralysis in 100 per cent of the controls, suggesting that variations in natural resistance do occur among normal monkeys, it was considered advisable to repeat these experiments once more with a larger number of animals. There was also the need for further investigation of some essential details which had hitherto been studied only imperfectly. Most important of these was the question as to the effectiveness of C treatment at various intervals following infection and the establishment of an optimum dosage of ascorbic acid. It remained also to be determined whether or not synthetic vitamin C possessed resistance-enhancing properties similar to that of the natural preparation.

Methods

The present communication deals with observations based on a total of 282 intracerebrally infected monkeys, treated at various intervals with various doses of vitamin C. Of these, 181 had received natural vitamin C and 101 either one of two different synthetic compounds. A total of 98 control monkeys, which were infected intracerebrally with corresponding amounts of virus and remained untreated, accompanied these tests. There is also included a group of 30 animals, infected by intranasal instillation of virus and treated with natural vitamin C, and 15 similarly infected controls. The virus used for the intracerebral tests was the Aycock strain which had been employed in our previous work. For the nasal infection the Rockefeller mixed virus was used.[1]

The general technique followed closely that outlined in detail in a previous paper (1). *Rhesus* monkeys weighing from 2000 to 3000 gm. were injected intracerebrally with amounts of virus (Aycock strain) ranging from 0.01 to 0.1 cc. (1 cc. of a 1:100 to 1:10 dilution) of a 10 per cent suspension. The intranasal infection was accomplished by instilling 1 cc. of a 5 per cent suspension of virus (R. M. V.) into each nostril and repeating this procedure 4 hours later. The treated animals received daily subcutaneous injections of vitamin C, the dosage varying from 5 to 100 mg., for a period of 2 weeks. Three different preparations of crystalline vitamin C were used in these tests. The first was a product extracted from natural sources (Merck), the second and third were synthetic preparations manufactured by either Merck and Company or Hoffmann-La Roche. Although all three preparations gave the identical reduction values for *l*-ascorbic acid with the indophenol reagent, they looked slightly different as far as the texture and color of the individual batches were concerned. The natural product, moreover, was said to contain approximately 2.5 per cent of unidentified impurities absent in either of the synthetic preparations.[2]

A total of twelve different series were run, ten of which represented intracerebral and two intranasal tests. The number of treated animals in each series varied from 10 to 49, the number of accompanying controls from 5 to 20. In some of these series different C products were used side by side in order to secure more strictly comparable data. The time at which vitamin C was first administered covered a range extending from the 1st to the 5th day of the infection, *i.e.*, until shortly before the onset of paralytic symptoms. All animals were carefully observed and a daily temperature record maintained for a period of from 3 to 4 weeks. In case of paralysis or death, an autopsy was made and the diagnosis confirmed by histological examination of the cord. Some of the surviving animals

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[1] This virus was kindly given to us by Dr. Albert B. Sabin of The Rockefeller Institute.

[2] We are indebted to Merck and Company for their cooperation in placing at our disposal generous amounts of their vitamin C preparations.

TABLE I

Vitamin C Therapy in Experimental Poliomyelitis

Series	Date	Amount of virus	Vitamin C	Time of first administration of vitamin C	Dose	Number of animals	Typical paralysis	Atypical paralysis*	No paralysis
	1936	*cc.*		*day of infection*	*mg.*				
1	Oct. 17	0.02	Natural (Merck)	1st	5	6	2	1	3
		"	" " "	1st	25	3	1	0	2
		"	" " "	2nd	5	6	3	0	3
		"	" " "	2nd	25	3	2	0	1
		"	" " "	3rd	5	6	0	2	4
		"	" " "	3rd	25	3	0	0	3
		"	—	—	—	9	5	1	3
2	Nov. 17	0.01	Natural (Merck)	1st	5	5	4	0	1
		"	" " "	1st	25	3	3	0	0
		"	" " "	2nd	5	6	3	1	2
		"	" " "	2nd	25	3	1	0	2
		"	" " "	3rd	5	6	2	0	4
		"	" " "	3rd	25	3	1	0	2
		"	—	—	—	8	7	0	1
3	Dec. 7	0.1	Synthetic (Merck)	1st	5	5	3	2	0
		"	" " "	3rd	5	6	3	2	1
		"	" " "	4th	5	5	4	1	0
		"	" " "	5th	5	6	4	2	0
		"	—	—	—	12	12	0	0
4	*1937* Jan. 16	0.05	Synthetic (Merck)	1st	5	10	10	0	0
		"	" " "	3rd	5	10	8	1	1
		"	" " "	5th	5	10	8	1	1
		"	" " "	5th	50	10	8	1	1
		"	—	—	—	20	20	0	0
5	Feb. 4	"	Natural (Merck)	1st	5	5	4	1	0
		"	" " "	1st	25	5	3	2	0
		"	" " "	3rd	5	5	3	0	2
		"	" " "	3rd	25	5	4	1	2
		"	" " "	5th	5	5	3	2	0
		"	" " "	5th	25	5	3	1	1

* Incubation period over 14 days.

TABLE I—*Concluded*

Series	Date	Amount of virus	Vitamin C	Time of first administration of vitamin C	Dose	Number of animals	Typical paralysis	Atypical Paralysis*	No paralysis
	1937	*cc.*		*day of infection*	*mg.*				
5	Feb. 4	0.05	Natural (Merck)	5th	50	5	1	2	2
		"	" "	5th	100	5	3	0	2
		"	Synthetic (Hoffmann-La Roche)	1st	5	5	2	1	2
		"	" "	1st	25	5	5	0	0
		"	—	—	—	11	11	0	0
6	Feb. 25	"	Natural (Merck)	5th	25	5	4	0	1
		"	" "	5th	50	5	3	1	1
		"	Synthetic (Hoffmann-La Roche)	3rd	5	5	3	0	2
		"	" "	3rd	25	5	4	0	1
		"	—	—	—	8	8	0	0
7	Mar. 22	"	Synthetic (Hoffmann-La Roche)	5th	5	5	3	2	0
		"	" "	5th	25	5	4	0	1
		"	" "	5th	50	5	4	0	1
		"	" "	5th	100	4	3	1	0
		"	—	—	—	5	5	0	0
8	Mar. 23	0.02	Natural (Merck)	3rd	5	10	6	1	3
		"	" "	3rd	25	10	8	0	2
		"	—	—	—	10	9	1	0
9	Apr. 26	0.05	Natural (Merck)	1st	5	15	12	0	3
		"	" "	1st	25	9	6	0	3
		"	" "	2nd	25	10	6	1	3
		"	" "	3rd	25	6	5	0	1
		"	" "	5th	5	4	3	0	1
		"	" "	5th	100	5	4	0	1
		"	—	—	—	10	9	0	1
10	May 24	"	Natural (Merck)	2nd	25	10	7	0	3
		"	—	—	—	5	5	0	0

were sacrificed, beginning with the 24th day, and their tissues examined for vitamin C content, others were kept until the termination of 1 to 1½ months.[3]

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[3] The results of the vitamin C titrations will be found in another paper (2).

The protocols of the individual series are given in Table I. A summary in which all intracerebrally infected animals are grouped together according to the kind and amount of vitamin C used will be found in Table II, while Table III lists the results obtained with treatment begun on different days of the infection. Table IV brings together the results obtained in the two intranasal series.

RESULTS

A study of Table I shows that in every one of the ten series, irrespective of the size of the infecting dose of virus, a definite though variable number of C-treated animals escaped paralysis.[4] Similar survivals among accompanying untreated controls occurred only in three series, two of the latter representing animals which had been infected with the smallest amount of virus, i.e., 0.02 and 0.01 cc., respectively. While there can therefore be no doubt that the absence of paralysis among C-treated animals was clearly significant in at least seven series in which all corresponding controls succumbed to the disease, the percentage of non-paralytic survivors in the three remaining series was considerably higher among the treated animals than among the controls. Thus, in series 1 we have surviving without paralysis 16 (59.2 per cent) of 27 treated animals against 3 (33.3 per cent) of 9 controls, in series 2, 11 (42.3 per cent) of 26 treated animals against 1 (12.5 per cent) of 8 controls, and in series 9, 12 (24.4 per cent) of 49 treated animals against 1 (10 per cent) of 10 controls. Obviously, the lighter infection favored the occurrence of non-paralytic survivors among both treated and untreated animals alike, but to a dissimilar extent.

Important differences in the efficacy of the natural and synthetic vitamin C preparations are revealed by Table II. Among a total of 181 infected monkeys, which had been treated with natural vitamin C, 58 (32 per cent) survived without paralysis, 16 (8.8 per cent) developed an atypical attack of poliomyelitis (incubation period longer than 2 weeks), and 107 (59.1 per cent) succumbed to the disease in typical fashion. On the other hand, among a total of 101 in-

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[4] It should be pointed out that a majority of the non-paralytic survivors pass through a distinct fever cycle during the infection, resembling an abortive attack of poliomyelitis, and that there are very few animals in which no rise in temperature is noted at any time.

fected monkeys which had been treated with synthetic vitamin C (Hoffmann-La Roche or Merck), 11 (10.8 per cent) survived without paralysis, 14 (13.8 per cent) showed an atypical attack of poliomyelitis, and 76 (75.2 per cent) succumbed to the disease in a typical manner. The above figures should be compared with the results obtained with control animals infected with the same amounts of virus which remained untreated. Of a total of 98 controls 5 (5.1 per cent) failed to show any paralysis, 2 (2 per cent) developed poliomyelitis after a

TABLE II

Comparison of the Effect of Natural and Synthetic Vitamin C in Experimental Poliomyelitis

Preparation of vitamin C	Dose	Number of animals	Result		
			Typical paralysis (incubation 1 to 14 days)	Atypical paralysis (incubation over 14 days)	No paralysis
	mg.				
Natural	100	10	7 (70 per cent)	0 (0 per cent)	3 (30 per cent)
(Merck)	50	10	4 (40 " ")	3 (30 " . ")	3 (30 " ")
	25	82	51 (62.1 " ")	5 (6 " ")	26 (31.7 " ")
	5	79	45 (56.9 " ")	8 (10.1 " ")	26 (32.9 " ")
		181	107 (59.1 per cent)	16 (8.8 per cent)	58 (32 per cent)
Synthetic	100	4	3 (75 per cent)	1 (25 per cent)	0 (0 per cent)
(Merck or	50	15	12 (80 " ")	1 (6.6 " ")	2 (13.3 " ")
Hoffmann-	25	15	13 (86.6 " ")	0 (0 " ")	2 (13.3 " ")
La Roche)	5	67	48 (71.6 " ")	12 (17.6 " ")	7 (10.4 " ")
		101	76 (75.2 per cent)	14 (13.8 per cent)	11 (10.8 per cent)
Controls (untreated)	—	98	91 (92.8 per cent)	2 (2 per cent)	5 (5.1 per cent)

prolonged incubation period, and 91 (92.8 per cent) succumbed to typical paralysis.

It is therefore evident that the percentage of non-paralytic survivors following treatment with natural vitamin C was about six times as large as that of the untreated controls. In the animals treated with synthetic vitamin C this percentage was only twice that of the controls. In both treated groups the percentage of animals developing atypical paralysis was approximately the same,

between 5 and 6 times as large as that observed among controls. However the difference between the two treated groups is again clearly demonstrated by the fact that treatment with natural vitamin C had reduced the incidence of typical paralysis by about one-third as compared with the controls (from 92.8 to 59.1 per cent), while the re-

Days of incubation period

▬ Duration of vitamin C administration.
▨ Interval between C administration and on-
   set of paralysis.   C.P-Complete paralysis
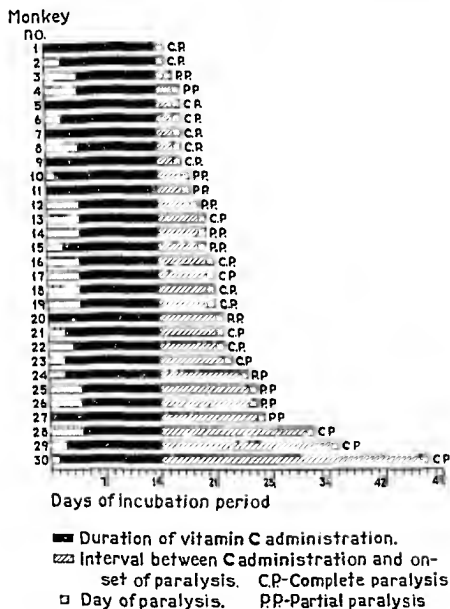▢ Day of paralysis.   P.P-Partial paralysis

CHART 1. Delayed paralysis in C-treated monkeys.

duction for the two synthetic preparations combined was less than one-fifth (from 92.8 to 75.2 per cent).

Detailed data concerning the delayed onset of paralysis in a total of 30 C-treated monkeys are given in Chart 1. It will be noted that the incubation period in these animals extended from a minimum of

15 days to a maximum of 47 days. Although these animals carried live virus in the central nervous system for long periods of time, there was no immunizing effect from such a carrier state as indicated by the fact that a majority developed eventually severe prostrating paralysis.

The figures given in Table II, even though listed according to dosage of vitamin C, do not lend themselves readily to a fair evaluation of the relative efficacy of different amounts of vitamin C since treatment with such doses was begun on various days of the disease. Caution should therefore be used in their interpretation. As they stand, however, there is very little difference in the percentage of non-paralytic survivors among the several groups treated with different amounts of natural vitamin C, the figures running from 30 per cent to 32.9 per cent. A similar uniformity may be observed in the results obtained following treatment with synthetic vitamin C, except that the figures for all doses are consistently lower, ranging from 0 per cent in a small group of 4 animals treated with 100 mg. to between 10.4 per cent and 13.3 per cent for the remaining groups.

A detailed analysis of our data according to the kind of vitamin C used, dosage employed and day of first administration is given in Table III. It becomes at once apparent that the number of animals in some of the subdivisions, particularly with the synthetic preparations, is too small to allow of a fair interpretation. However it would seem that the administration of 5 or 25 mg. of natural vitamin C, begun on the day of infection, produces about the same result, i.e., between 22.5 per cent and 25 per cent of the animals surviving without paralysis. When the first administration of vitamin C is delayed until the 2nd and 3rd day of the infection, considerably higher percentages of non-paralytic survivors are observed, especially among the animals injected with 5 mg. Thus it would appear that between 41.6 per cent and 48 per cent of the animals treated with 5 mg. and between 34.6 per cent and 38.4 per cent of those treated with 25 mg. of vitamin C escape paralysis at that time. We are rather inclined to the belief that the seemingly better figures are more apparent than real and do not indicate a real improvement of C treatment at the later stages of the disease. It should be remembered that a great portion of the animals listed in these two categories were taken from series 1 and 2 in which very small doses of virus were used,

resulting in an abnormally high accumulation of non-paralytic survivors among both treated animals and controls. If an allowance were made for this fact and the figures brought into accord with those of the other categories, the percentage of non-paralytic survivors among animals treated at this time would not materially exceed 30 per cent to 35 per cent. However there would still remain a slight advantage in favor of the animals treated with 5 mg. over those treated with 25 mg. More conclusive results are obtained with animals in which vitamin C treatment was not begun until the 5th day of the disease. At this time distinctly larger doses of vitamin C are apparently required to obtain significant results. Thus the percentage of non-paralytic survivors increases progressively from 11.1 per cent (5 mg.) to 20 per cent (25 mg.), and reaches a maximum of 30 per cent when doses of 50 to 100 mg. are used.

Much less regular results are observed in animals treated with the two synthetic preparations, due, in part at least, to the small number of animals in some of the subdivisions. However, here again it would seem that better figures are obtained with animals treated on the 3rd day of the disease (about 20 per cent) than with those in which treatment was begun on the day of infection (0 to 5 per cent). While the percentage of non-paralytic survivors shows also a tendency to drop when treatment is delayed until the 5th day of the disease, there is no unmistakable correlation to the size of the dose employed.

In comparing the results obtained with natural and with synthetic vitamin C it can safely be stated that, irrespective of the interval that had elapsed since infection, the percentage of non-paralytic survivors among animals treated with natural vitamin C, dose for dose, is distinctly higher than that observed for animals which had received either of the two synthetic preparations. Good results can still be obtained with natural vitamin C until the 3rd day, and fair results, with adequate dosage, on the 5th day of the disease. Treatment with synthetic vitamin C, on the other hand, is so irregular, on the whole, and so inefficient that it is doubtful whether the data will bear critical examination, except for the suggestion of a slight reduction in the severity of the disease as compared with untreated controls.

We have run only two series in which the effect of natural vitamin

C was studied on the course of experimental poliomyelitis induced by nasal instillation of the virus. In all animals treatment was begun

TABLE III

Effect of Various Doses of Natural and Synthetic Vitamin C When Administered on Different Days of Infection

Preparation of vitamin C	Day of infection	Dose	Number of animals	Result		
				Typical paralysis (incubation 1 to 14 days)	Atypical paralysis (incubation over 14 days)	No paralysis
		mg.				
Natural	1st	5	31	22	2	7 (22.5 per cent)
(Merck)		25	20	13	2	5 (25 " ")
	2nd	5	12	6	1	5 (41.6 " ")
		25	26	16	1	9 (34.6 " ")
	3rd	5	27	11	3	13 (48 " ")
		25	26	15	1	10 (38.4 " ")
	5th	5	9	6	2	1 (11.1 " ")
		25	10	7	1	2 (20 " ")
		50	10	4	3	3 (30 " ")
		100	10	7	0	3 (30 " ")
			181	107 (59.1 per cent)	16 (8.8 per cent)	58 (32 per cent)
Synthetic	1st	5	20	15	3	2 (5 per cent)
(Merck or Hoff-		25	5	5	0	0 (0 " ")
mann-La	3rd	5	21	14	3	4 (19 " ")
Roche)		25	5	4	0	1 (20 " ")
	4th	5	26	19	6	1 (3.8 " ")
	and	25	5	4	0	1 (20 " ")
	5th	50	15	12	1	2 (13.3 " ")
		100	4	3	1	0 (0 " ")
			101	76 (75.2 per cent)	14 (13.8 per cent)	11 (10.8 per cent)
Controls (untreated)			98	91 (92.8 per cent)	2 (2 per cent)	5 (5.1 per cent)

on the day of infection and maintained, as usual, for a period of 2 weeks. The results are given in Table IV. It will be noted that all

10 animals injected with 5 mg. of vitamin C succumbed to the disease, as did 9 of 10 animals injected with 25 mg. On the other hand, there were 9 animals that escaped paralysis among a group of 10 monkeys which had received doses of 50 to 100 mg. of vitamin C. Since all of the 15 untreated controls which accompanied these tests developed paralysis, it would seem that very encouraging results may be obtained with vitamin C treatment in this type of infection, although much larger doses of ascorbic acid are evidently required than for intracerebral tests. However it is planned to continue this work on a larger scale before valid conclusions can be drawn.

TABLE IV

Effect of Natural Vitamin C in Experimental Poliomyelitis
(Intranasal Infection)

Series	Dose of vitamin C	Number of animals	Result		
			Typical paralysis (incubation 1 to 14 days)	Atypical paralysis (incubation over 14 days)	No paralysis
	mg.				
11	5	10	10	0	0
	25	10	8	1	1
	—	10	10	0	0
12	50	5	1	0	4
	100	5	0	0	5
	—	5	5*	0	0

* One of the 5 controls listed in series 12 as developing typical paralysis showed no distinct paralysis of the extremities but a very marked facial paralysis.

It seems to us that the significance of results obtained in therapeutic studies, such as the one here reported, lies not so much in isolated observations of success, or lack of success, obtained in one or several small experimental series, but that their intrinsic value is considerably strengthened if such results can be duplicated in prolonged investigations. For this reason it is interesting to compare the data obtained in this study with those published in our previous report, even though the two studies differ substantially among themselves in important details such as dosage of vitamin C, time of ad-

ministration, and amount of virus used.   It will be noted from Table V that the two sets of figures are in exceptionally good agreement. Thus the average percentage of intracerebrally infected monkeys which escape paralysis following treatment with natural vitamin C stands between 30.6 per cent and 32 per cent, while only between 5.1 per cent and 5.2 per cent of the untreated controls fail to develop paralytic symptoms.   These data are now based on a total of 243 (181 plus 62) treated monkeys and 136 (38 plus 98) controls, leaving little doubt as to the accuracy of the observations.   However, further detailed statistical analysis of the two sets of figures shows

TABLE V

Effect of Natural Vitamin C in Experimental Poliomyelitis
(Comparison of Data Obtained in This Study with Data Previously Published)

Experimental data	Type of animal	Number of animals	Result		
			Typical paralysis	Atypical paralysis	No paralysis
Present data...	C-treated	181	107 (59.1 per cent)	16 (8.8 per cent)	58 (32 per cent)
Previous data...	" "	62	38 (61.1 " ")	5 (8 " ")	19 (30.6 " ")
Present data...	Controls	98	91 (92.8 " ")	2 (2 " ")	5 (5.1 " ")
Previous data...	"	38	34 (89.4 " ")	2 (5.2 " ")	2 (5.2 " ")

that the percentage of animals treated with 5 mg. alone has not inconsiderably dropped from that reported heretofore.   Accidental features, connected with the limited number of animals used, may have caused the first figure to be unduly high.   There is also a possibility that different preparations of natural vitamin C may differ conspicuously in the degree of their purity or the extent to which other extraneous substances may modify the therapeutic effect.

DISCUSSION

The data presented in this paper, which are based on a number of experimental animals almost thrice as large as previously reported,

leave no doubt that the administration of natural vitamin C during the incubation period of experimental poliomyelitis is followed by a definite alteration in the severity of the disease. This is indicated by the fact that about six times as many treated animals escape paralysis (32 per cent) as do corresponding controls (5.1 per cent). Conversely, the incidence of typical paralysis stands at 59.1 per cent among the treated monkeys as compared with 92.8 per cent among untreated control animals. The given percentages are essentially identical with those previously published by us, *i.e.*, there has been neither a significant reduction nor a conspicuous increase, when the data are taken as a whole. These figures demonstrate clearly the possibilities as well as the limitations of therapy with natural vitamin C in experimental poliomyelitis.

Different results were obtained when synthetic vitamin C was used. In contrast to the above figures, we find only about twice as many non-paralytic survivors in animals treated with this substance (10.8 per cent) as among the controls, and the incidence of typical paralysis stands at 75.2 per cent in this treated group.

It would appear, therefore, that the measure of therapeutic success depends upon certain variables which, in order of significance, may be listed as follows: First, the kind of vitamin C; second, the dosage of vitamin C; third, the rapidity of the infection as determined by the amount of virus and type of strain; and fourth, the time of first administration of vitamin C. As a fifth factor, equally as important as the other four, we should mention differences in individual response from monkey to monkey which necessitate running large series of animals if significant results are to be obtained.

The markedly superior therapeutic efficacy of natural vitamin C, as contrasted with the synthetic preparations, is surprising, since we found but little discrepancy in virucidal action between the two in *in vitro* experiments. It is realized that the assumption of a real difference between the two kinds of products will find little favor with the biochemist who considers them as identical, except for possible impurities in ascorbic acid extracted from natural sources. Similarly, clinical experience has proven that both preparations possess the same antiscorbutic properties in man and animals. However, recent data tend to show that the natural substrate of vitamin C

contains additional factors, absent in the chemically pure synthetic product, which may be present in variable amounts in natural preparations. We are alluding here to the so called P factor discovered by Szent-Györgyi (3) which exerts an antipurpuric effect and the so called J factor of von Euler and Malmberg (4) which apparently protects against pneumococcic infection in guinea pigs. Divergent results between synthetic and natural vitamin C have also been described by Havas and Gal (5) who found that the same concentration of ascorbic acid either stimulated or inhibited the growth of seedlings, depending upon whether the natural or synthetic product were employed. There is finally a possibility that mineral impurities of the natural product may serve as activators (6).

The problem of dosage is complicated by the fact that the largest amounts of ascorbic acid are not always necessarily optimal. This is clearly shown by a series of recent observations in widely differing fields all of which demonstrate that excessive amounts of vitamin C may bring about a reversal of the effect noticed with smaller doses (7). While our data are still too limited to make deductions certain, it would appear that during the first few days of the infection doses of 5 to 25 mg. produce an optimum therapeutic effect and that definitely larger doses, ranging from 50 to 100 mg., are required for treatment which is delayed until shortly before the expected onset of paralytic symptoms. For the same reason it can easily be understood that a faster progressing infection such as that induced by the R.M.V. strain responds only to doses of vitamin C definitely larger than those which are sufficient to halt the slower infection with the Aycock virus. The different dosage required for the two types of infections can certainly more easily be explained on that basis than by assuming differences in type of strain or mode of infection. The size of the effective dose of vitamin C therefore seems to be directly proportional to the speed of the infection and the stage at which the infectious process is advanced.

It is altogether impossible to say why the therapeutic effect should be so irregular, varying as it does, series for series, among different animals even though the average percentage of non-paralytic survivors remains fairly well fixed. The nature of this individual factor at present is wholly unknown. Obviously there are other conditions

which determine the extent to which vitamin C proves of benefit to the monkey. From the results of our vitamin C titrations of various monkey tissues it would seem possible that the protected animals may utilize vitamin C more effectively than the unprotected ones (2). Whether the degree of this utilization depends upon an unknown endocrine factor is an open question.

The mechanism by which vitamin C accomplishes its therapeutic effect still remains obscure. Obviously the simplest explanation would be to assume that ascorbic acid, not unlike a chemotherapeutic agent, inactivates poliomyelitis virus directly in the central nervous system, particularly since the latter can be shown to store this substance. Such inactivation would then be largely non-specific, since ascorbic acid, properly adjusted for pH, has proven capable of inactivating by direct contact *in vitro* every toxin and virus that has been investigated (8). However in spite of the frequent occurrence of such crude "antiviral" and "antitoxic" phenomena *in vitro*, therapeutic results with vitamin C have been obtained only in a minority of infectious diseases, among which tuberculosis (9) and diphtheria (10) are outstanding. This would suggest that vitamin C may conceivably act in a more specialized manner in certain types of tissue injury by enhancing the mechanism of natural defense. An example of such action is found in the observation that mice injected with vitamin C are protected against fatal doses of acetonitril (11), a poison to which they are seasonally more susceptible in the summer than in the winter (12). Evidence supporting the supposition that poliomyelitis virus is not destroyed directly by the vitamin *in vivo* comes from our observation that a considerable number of treated animals develop paralysis after greatly prolonged incubation periods, *i.e.*, at a time following discontinuance of C treatment when the artificially raised C levels have dropped again to their normal values (see Chart 1). In such cases the virus was clearly not killed but kept in check possibly as long as the cells maintained a high concentration of vitamin C. The irregularity of the therapeutic effect, so characteristic of all our experiments, would also plead in favor of a complex reaction, mediated through the cells, rather than for a direct virucidal action.

It has been pointed out by competent workers in vitamin research

that the chemist has superseded the pharmacologist who must surely recognize the probability that substances of an intensive physiological activity may exercise therapeutic functions that may seem comparatively remote from their normal nutritional mode of action (13). And such phrases as "conditioned vitamin deficiencies" or "toxic avitaminoses" continue to appear in the literature devoted to this new and promising field.   If we are to accept vitamin C, therefore, as a therapeutic agent on the basis of its established physiological action rather than of its normal rôle in nutrition, the question arises in what way the anti-infectious effect of ascorbic acid in poliomyelitis can be correlated with its biochemical activity.   Since it is commonly suspected that one of the functions of vitamin C is to regulate the oxidation-reduction potential of cellular respiration (14), it becomes relatively easy to imagine that an increased supply of the vitamin, which has a tendency to diminish during the infection, serves to maintain the oxidation-reduction system of nerve cells at a level at which the oxylabile virus is restrained from intracellular propagation. While this suggestion is still entirely hypothetical, it is in harmony with the observations of Brodie and Wortis (15) who have demonstrated a diminution in the oxygen consumption rate of nerve tissue infected with poliomyelitis virus.

In the light of these remarks it will readily be understood why vitamin C may be useful as an auxiliary therapeutic agent in certain infectious diseases which have little in common with the classical symptoms of frank scurvy.   As far as poliomyelitis is concerned, the rationale of such treatment, at present, is predicated only by the experimental data given in this paper.   It would be considerably strengthened by the establishment of a causal relationship between susceptibility to the disease and faulty C metabolism.   Such demonstration would have to come from clinical studies and from epidemiological observations involving a correlation between the extent of the so called prescorbutic state in different age groups, latitudes, and seasons, on the one hand, and the distribution of cases of poliomyelitis, on the other.   It is not likely, however, that abnormally low C levels are summarily produced by a low intake of vitamin C, except in regions where the content of vitamin C in food is notoriously inadequate, such as in some rural districts of northern countries (16).

It would seem rather as if the C requirements of exceptional individuals at certain times are greater than can be met and that crises occur, precipitated by a combination of environmental factors such as light, fatigue, intestinal disturbances, and other unknown agencies, which may facilitate central nervous system invasion of the ubiquitous virus.

SUMMARY AND CONCLUSIONS

1. A group of 181 monkeys were infected intracerebrally with amounts of virus ranging from 0.01 to 0.05 cc. of a 10 per cent virus suspension. At different intervals following infection treatment of these animals was begun with daily injections of 5 to 100 mg. of natural vitamin C for a period of 2 weeks. Of 89 monkeys treated on the 1st or 2nd day of infection 26 (29.2 per cent) survived without showing any evidence of paralysis; of 53 monkeys treated on the 3rd day of the infection 23 (43.3 per cent) survived without showing any evidence of paralysis; of 39 monkeys treated on the 5th day of the infection 9 (23 per cent) survived without showing any evidence of paralysis.

2. A group of 101 monkeys were infected intracerebrally with amounts of virus ranging from 0.05 to 0.1 cc. of a 10 per cent virus suspension. At different intervals following infection treatment of these animals was begun with daily injections of 5 to 100 mg. of synthetic vitamin C for a period of 2 weeks. Of 25 monkeys treated on the 1st day of infection 2 (8 per cent) survived without showing any evidence of paralysis; of 26 monkeys treated on the 3rd day of the infection 5 (19.2 per cent) survived without showing any evidence of paralysis; of 50 monkeys treated on the 4th and 5th day of the infection 4 (8 per cent) survived without showing any evidence of paralysis.

3. The above two groups of treated animals were accompanied by a group of 98 control monkeys which were infected intracerebrally with the same amounts of virus and remained untreated. In this group there were 5 (5.1 per cent) animals which survived without showing any evidence of paralysis.

4. The figures, taken as a whole, show that among 181 monkeys treated with natural vitamin C 58 (32 per cent) survived without paralysis, and among 101 monkeys treated with synthetic vitamin C

11 (10.8 per cent) survived without paralysis. In comparing the percentage of non-paralytic survivors of the two treated groups with that of the untreated controls (5.1 per cent) it is found that about six times as many animals escaped paralysis following treatment with natural vitamin C as did the corresponding controls. In the group of animals treated with synthetic vitamin C, on the other hand, there were only about twice as many non-paralytic survivors as among the controls.

5. The results obtained in this investigation, as far as they are concerned with the therapeutic effect of natural vitamin C in experimental poliomyelitis, are in close agreement with the data previously published.

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VITAMIN C CONTENT OF MONKEY TISSUES IN EXPERIMENTAL POLIOMYELITIS*

By CLAUS W. JUNGEBLUT, M.D., AND ROSE R. FEINER

(From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York)

During the past few years much attention has been given to the study of vitamin C metabolism in health and disease. What has rendered this problem so attractive to laboratory workers and clinicians alike is the fact that vitamin C is not only significant as an accessory factor in nutrition, absence of which causes characteristic degenerative lesions in various tissues, but that ascorbic acid has come to be recognized as a physiological substance of great biochemical potency which seems to play an important part in the regulation of certain cell functions concerned with the mechanism of normal defense against microbial injury (1). This is borne out by numerous observations on increased susceptibility to bacterial infections and intoxications in C-deficient animals and proven more directly by the discovery that the vitamin C stores are often severely depleted during the progress of an infectious process. The latter data are based on vitamin C assays of the tissues of experimentally infected animals, as well as on the demonstration of C deficiencies in patients suffering from infectious diseases as determined by the urinary excretion following oral administration of large doses of ascorbic acid.

As might be expected, attempts have been made to compensate for this loss of vitamin C by increasing the intake; and evidence is beginning to accumulate which suggests that the administration of vitamin C during the course of certain infections and intoxications in man and animals may be followed by a reduction in severity of the pathological process. It is an open question whether such resistance-enhancing effects are due to an indirect stimulation of the physiological defense

mechanism of the cells or whether ascorbic acid is capable of destroy-ing directly certain bacteria, toxins, and viruses *in vivo*, as has clearly been demonstrated to occur when such infectious agents are brought together with the vitamin *in vitro*.   While changes in vitamin C levels, occurring as they do in the wake of many infectious processes, do not necessarily signify any causal relationship to susceptibility, it has seemed possible that vitamin C metabolism may assume a particular significance in those infectious diseases which are normally charac-terized by wide variations in individual resistance.   This would seem to apply especially if such infections or intoxications should prove to respond favorably to therapeutic doses of ascorbic acid as appears to be true for diphtheria, tuberculosis, and poliomyelitis (2), to quote only the best studied examples.   It is for this reason that we have un-dertaken a detailed investigation of the vitamin C content of monkey tissues in experimental poliomyelitis, the object of this study being to compare the vitamin C levels of various representative tissues in normal monkeys with those of poliomyelitic monkeys and of infected animals in which an attempt had been made to lessen the severity of the infection by parenteral administration of vitamin C during the incubation period of the disease (2).

Methods

The work was planned to provide as fair a basis of comparison as possible between the vitamin C levels of normal and poliomyelitic monkeys which had received no injections of ascorbic acid and those of normal and infected animals which had been injected daily for 2 weeks with doses of ascorbic acid ranging from 5 to 100 mg.   All animals were kept on the same general balanced dietary régime, which consisted of rice, bread, and milk and included an ample amount of bananas, oranges, and lettuce as a source of vitamin C.   While the quality of the diet was therefore roughly adequate, no strict control of the quantitative individual intake of food could be obtained since the monkeys were sitting in groups of 10 to 15 in large cages.   All monkeys were of approximately similar weight, ranging from 2300 to 2800 gm., and were sexually immature.   The tissues studied were particu-larly brain, cord, and suprarenal; but we have also run numerous titrations of other organs such as liver, spleen, gonads, kidney, and intestines.   Few tests were done with blood and spinal fluid since it was soon discovered that these fluids contained none or so little vitamin C that the amounts could not be accu-rately measured.   Normal monkeys were sacrificed from time to time together with poliomyelitic monkeys so as to control any variables that might result from

the fact that this study extended over a period of several months (January to May) and that the animals were received from different shipments. Poliomyelitic monkeys, if prostrated, were killed at the height of paralysis in order to exclude any gross error due to inanition.

The monkeys (*Macacus rhesus*) were killed by intracardiac injection of air, and the tissues immediately removed and titrated for vitamin C according to the modified method of Bessey and King (3). This method measures only the reduced vitamin C present in the tissues, and is based on the fact that vitamin C, in strongly acidic solutions (of pH 2-3), will reduce very rapidly the indophenol reagent to its colorless base. The dye is blue in alkaline and pink in acid solution; the end-point of the titration is reached when the acid extract turns a pink color. The tissues are weighed accurately and then ground with sand in a mixture of 8 per cent trichloracetic and 2 per cent metaphosphoric acid in a glass mortar until a very fine homogeneous suspension is obtained. This suspension is centrifuged and the supernatant poured into a volumetric flask. The residue is again extracted with acid and centrifuged; the supernatants are then brought up to a volume of 25 or 50 cc., depending on the expected concentration of C in the tissues. Aliquot portions of the extract are titrated against sodium 2-6 dichlorobenzenone indophenol (Eastman Kodak P 3463). The dye is usually made up so that 1 cc. of the solution is the equivalent of 0.1 to 0.3 mg. of vitamin C. The indicator is standardized against an acid solution of crystalline vitamin C, which in turn has been standardized against a 0.01 N solution of iodine. The titrations are completed within about 30 seconds and the first pink color that appears is taken as the end-point. A blank of the acid mixture alone is always run for any given volume of extract. Other substances in tissue extracts will reduce the indicator even in strongly acid solution, although more slowly than vitamin C (4). For this reason a rapid end-point (5 to 30 seconds) is considered fairly accurate; however difficulties are often encountered when tissue extracts (*e.g.*, liver, kidney) contain substances which reduce the dye almost as rapidly as does vitamin C. The end-point is such that although it is rapidly fading, another drop of indicator is not immediately reduced by the extract as it would be if vitamin C were still present. The results, with correction for the blank, are calculated as mg. of vitamin C per gm. of tissue. The experimental error lies between 5 and 10 per cent. While the use of the indophenol reagent, therefore, may not give absolute values for the C content of tissues, we agree with others that it is accurate enough for comparative purposes, especially since the titration values against the dye are found to correlate closely with the values obtained by the biological assay method (5).[1]

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[1] Reversibly oxidized vitamin C may be brought back to the reduced state by the passage of H_2S through the solution for a length of time with subsequent removal of the H_2S by N or CO_2. Since substances other than dehydroascorbic acid may be reduced by H_2S and included in the titration, and since the routine

A total of 123 monkeys was killed for tissue titration.   This number is made up of four main groups which are listed as follows: (1) normal monkeys which had not received any vitamin C parenterally, (2) normal monkeys which had been injected subcutaneously with 5 or 25 mg. of ascorbic acid daily for a period of 2 weeks, (3) control monkeys which had not received vitamin C and showed various degrees of paralysis following intracerebral inoculation with poliomyelitis virus, and (4) vitamin C-treated monkeys which had received subcutaneous injections of 5 to 100 mg. of ascorbic acid daily for a period of 2 weeks following intracerebral infection with poliomyelitis virus.   This last group is further subdivided into monkeys which survived without paralysis following C treatment and those which succumbed to the disease in spite of treatment.   The results obtained in the several groups of monkeys will now be discussed individually.

Group 1.—Normal monkeys.   There were 5 normal monkeys in this group.   These were sacrificed from time to time during the course of this investigation after being kept on the ordinary feeding schedule for a period of at least 2 weeks.   The average C levels found in the central nervous system and suprarenals, expressed in mg. per gm. of tissue, were as follows: 0.08 for brain, 0.07 for cord, and 0.43 for suprarenal.

Group 2.—Normal C-prepared monkeys.   This group included 3 monkeys all of which were sacrificed on the day following the last injection of vitamin C.   The average C levels found in nervous tissue and the suprarenals of these animals were markedly higher than those observed for normal monkeys, *i.e.*, 0.15 for brain, 0.10 for cord, and 0.75 for suprarenal.

Group 3.—Poliomyelitic control monkeys.   This group comprised a total of 24 animals serving as untreated controls for vitamin C-treated monkeys which had been infected simultaneously.   Of these 24 animals 11 were completely prostrated by quadriplegia; the remaining

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use of this method was not practical we have run only a few such tests.   In those instances where this method was used we found no changes in our values that could not be accounted for by probable errors in technique.   It seems, therefore, likely that most of the vitamin was present in these tissues in the reduced form.

13 were partially paralyzed to various extents. The 11 completely paralyzed animals were killed at the height of paralysis and the 13 partially paralyzed animals at different stages of their convalescence, which varied from 3 to 4 weeks (7 animals) to 6 to 10 weeks (6 animals) following the onset of paralysis. The C levels found in animals killed at the height of paralysis as well as in the early stage of convalescence were uniformly low, average values of 0.07 and 0.06 being obtained for brain, 0.05 and 0.07 for cord, and 0.27 for suprarenal. These figures were slightly below those of corresponding normal monkey tissues. Somewhat higher average figures were observed in animals which were allowed to recover up to 10 weeks, *i.e.*, 0.13 for brain, 0.10 for cord, and 0.53 for suprarenal. It will be noted that these values were slightly above the C levels of normal monkeys.

Group 4. C-treated infected monkeys. This group is by far the largest, including a total of 91 monkeys in which an attempt had been made to influence the course of experimental poliomyelitis by parenteral administration of vitamin C during the incubation period. The total number of animals in this group is divided into two subgroups, the first subgroup representing 58 animals which developed paralysis in spite of treatment, while the second subgroup contained 33 animals which had survived without showing any evidence of paralytic symptoms.

The paralyzed animals, if prostrated by quadriplegia, were killed at the height of paralysis (35 animals) and if partially paralyzed were sacrificed at 3 to 4 weeks (19 animals) or at 5 to 10 weeks (4 animals) following the onset of paralysis. Titration of the tissues of these C-treated paralyzed animals showed average C values distinctly higher than those observed for untreated poliomyelitic controls, running from 0.13 to 0.14 for brain, from 0.09 to 0.11 for cord, and from 0.51 to 0.79 for suprarenal. These values were therefore either very slightly below or practically identical with the levels of normal C-prepared monkeys. There was little if any secondary rise in the figures concomitant with the progress of convalescence in this group, probably because the initial values were already high.

Interesting results were obtained in the second subgroup with C-treated animals which had escaped paralysis. These animals were sacrificed either between the 3rd and 4th week of survival following

the day of infection (21 animals) or as late as 6 weeks thereafter (12 animals). The average figures for those killed at the earlier period showed unusually high C levels in all tissues examined, *i.e.*, 0.16 for brain, 0.13 for cord, and 0.90 for suprarenal. However, when such animals had been permitted to live until 6 weeks after infection, these hypernormal values had again returned to figures which were only slightly above the normal average, *i.e.*, 0.13 in brain, 0.09 in cord, and 0.44 in suprarenal.

1. Normal monkeys
2. Poliomyelitic monkeys (height of paralysis)
3. Poliomyelitic monkeys (late convalescence)
4. C-prepared normal monkeys
5. C-treated infected monkeys (height of paralysis)
6. C-treated infected monkeys (late convalescence)
7. C-treated infected monkeys (3rd to 4th week of non-paralytic survival)
8. C-treated infected monkeys (6th week of non-paralytic survival)

CHART 1. Vitamin C content of monkey tissues in experimental poliomyelitis.

A summary of these titrations will be found in Table I, while Chart 1 gives a comparison of the C levels, tissue for tissue, in the different groups of animals.

As was mentioned before, we have also run numerous C titrations on tissues other than the central nervous system and the suprarenals. These determinations have yielded results, closely comparable to those reported above, *i.e.*, high or low levels of C in the brain, cord, and the suprarenals were usually reflected by high or low levels in the spleen, liver, gonads, and intestines. These data are therefore not reproduced

TABLE I

Vitamin C Content in the Brain, Cord, and Suprarenal of Normal and Poliomyelitic Monkeys (with and without Parenteral C-Administration)

Group	Type of animal	Number of animals	Brain		Cord		Suprarenal	
			Median range per gm.	Average per gm.	Median range per gm.	Average per gm.	Median range per gm.	Average per gm.
			mg.	mg.	mg.	mg.	mg.	mg.
1	Normal monkeys	5	0.04-0.11	0.08	0.05-0.10	0.07	0.25-0.69	0.43
2	Normal monkeys, C-prepared	3	0.15	0.15	0.09-0.10	0.10	0.66-0.86	0.75
3	Poliomyelitic control monkeys							
	Killed at height of paralysis	11	0.05-0.10	0.07	0.04-0.07	0.05	0.11-0.59	0.27
	Killed during early convalescence	7	0.04-0.09	0.06	0.05-0.10	0.07	0.09-0.40	0.27
	Killed during late convalescence	6	0.07-0.15	0.13	0.08-0.12	0.10	0.19-0 65	0.53
4	C-treated infected monkeys							
Subgroup 1	Killed at height of paralysis	35	0.08-0.16	0.13	0.08-0.11	0.09	0.52-1.04	0.79
	Killed during early convalescence	19	0.11-0.16	0.14	0.09-0.16	0.11	0.35-0.78	0.61
	Killed during late convalescence	4	0 09-0.17	0.13	0.07-0.12	0.10	0.18-0.83	0.51
Subgroup 2	Killed during 3rd to 4th wk. of non-paralytic survival	21	0.12-0 22	0.16	0.10-0.16	0.13	0.54-1.25	0.90
	Killed at 6th wk. of non-paralytic survival	12	0.11-0.16	0.13	0.08-0.11	0.09	0.24-0.86	0.44

in detail. However, we are giving in Table II and in Chart 2 the results of the titration of all tissues examined for normal monkeys and

TABLE II

Vitamin C Content of the Tissues of Normal and Vitamin C-Prepared Monkeys

| Type of animal	Suprarenal per gm.	Gonad per gm.	Spleen per gm.	Liver per gm.	Brain per gm.	Cord per gm.	Intestine per gm.	Kidney per gm.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Normal..........................	0.57	0.19♂	0.21	0.06	0.08	0.05	0.09	0.02
"	0.25	0.50♂	0.11	0.10	0.09	0.06	0.04	0.02
"	0.69	—	0.32	0.10	0.11	0.10	0.06	0.03
"	0.27	0.23♂	0.14	0.05	0.07	0.09	0.07	0.02
Normal C-prepared (5 mg.)........	0.66	0.53♂	0.41	0.16	0.15	0.10	0.21	0.05
Normal C-prepared (25 mg.).......	0.75	0.64♀	0.47	0.27	0.15	0.10	0.15	0.07
" " " " "	0.86	0.25♀	0.31	0.17	0.15	0.09	0.18	0.07
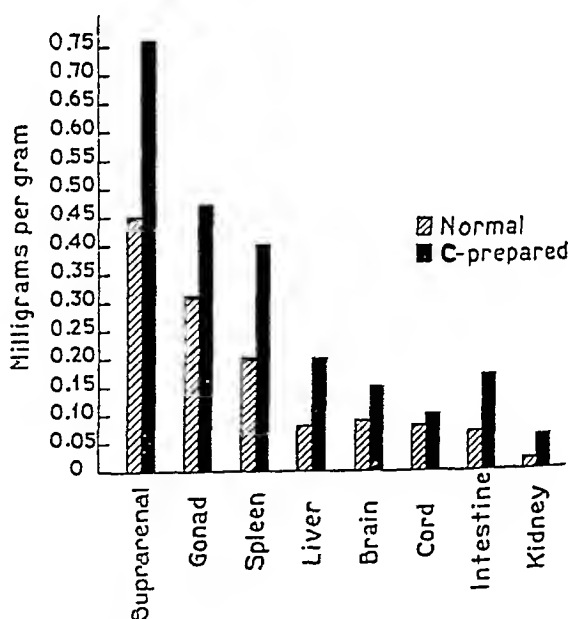
CHART 2. Vitamin C content of the tissues of normal and vitamin C-prepared monkeys.

normal C-prepared monkeys because they demonstrate clearly the increase in all values following preparation with vitamin C. These

increases are most marked in the liver, kidney; and intestine, and are followed by those in the spleen and brain. The cord seems to participate only to a very modest extent in this process of C assimilation.

DISCUSSION

The data presented in this paper offer information on the concentrations of vitamin C that are present in various tissues of normal *rhesus* monkeys and the extent to which such concentrations are subject to alteration by the parenteral administration of ascorbic acid. They are also helpful in explaining the results obtained with vitamin C therapy in experimental poliomyelitis.

It is well known that different animal species, on the basis of their susceptibility to experimental scurvy, may be divided into those which are capable of synthesizing their own vitamin C and those which depend upon dietary intake to supply this nutritional factor. There is well documented evidence to show that species of the first category, such as rats, rabbits, dogs, cats, and cattle, carry higher C levels in all tissues than man and the guinea pig, which belong to the second category (6). Although the monkey's susceptibility to experimental scurvy is well established, figures relating to the C content of monkey tissues, with the exception of the blood (7), have been wanting. The results of our titrations show that the C levels of the monkey fall in line with those of other non-synthesizing species. If the brain or suprarenal are taken as representative organs, the following series of C values, expressed in mg. per gm. of tissue, is therefore obtained: rat: 0.30 mg. (brain), 3 to 6 mg. (suprarenal); rabbit: 0.20 mg. (brain), 2 to 3 mg. (suprarenal); cat: 0.20 mg. (brain), 1 mg. (suprarenal); dog: 0.19 mg. (brain), 0.9 mg. (suprarenal); guinea pig: 0.14 mg. (brain), 0.3 to 0.7 mg. (suprarenal); man: 0.15 mg. (brain), 0.5 mg. (suprarenal); and monkey: 0.08 mg. (brain), 0.4 mg. (suprarenal). The values are slightly higher in very young and slightly lower in very old animals. Similar ratios are found in other tissues.

Since the amount of vitamin C found in the tissues depends upon the amount of ascorbic acid taken in, both orally and parenterally, C titrations must always be interpreted in terms of dietary intake. How sensitive an indicator the tissue C level is of variations in diet, other factors remaining constant, was forcibly brought to our attention in

this work.   For a short time some monkeys were fed more oranges than usual, with the result that the C values ran significantly higher. On the other hand, some monkeys which were sacrificed shortly after having been received from the dealer, showed considerably lower C levels in all tissues.   These animals were therefore excluded from tabulation in this study.   We have also found some evidence for the presence of a slight seasonal increase in tissue C levels of all monkeys, normal, treated, and controls, beginning with the month of June. For this reason we have omitted all titrations carried out after that date.   Even when due allowance has been made for variables which might affect the titration values, there still remains an extraordinary variability in the figures for animals within a given group, particularly in the suprarenal, as indicated by the extent of the so called median range from which the averages were computed.

The data obtained in this study have an important bearing on the nature of the therapeutic effect of vitamin C in experimental poliomyelitis.   There is some indication that the amounts of C present in nervous tissues and in the suprarenal tend to diminish slightly during the progress of the infection.   This is shown more clearly by contrasting the C levels of normal monkeys with those of poliomyelitic controls at the height of paralysis or during early convalescence than by a comparison of the figures obtained in normal and paralyzed animals which carried an additional load of vitamin C.   As far as the suprarenal is concerned, this loss of C during the disease is in accord with scattered preliminary observations previously reported (8).   However, it appears that the loss of C that occurs during poliomyelitic infection in the monkey is not nearly as striking as the C depletion observed in diphtheritic intoxication (9).   In favor of the assumption that the lower C levels in paralytic monkeys are due to a temporary derangement of C metabolism produced by the disease and not referable to chance factors is the fact that poliomyelitic monkeys show a distinct tendency for a return to normal and even slightly hypernormal C levels as convalescence progresses.   It is difficult, of course, to exclude with certainty that this secondary increase may not be the result of better adjustment to laboratory conditions.   However, the data recently published by Torrance (10), who observed a marked increase in C content of the suprarenal in guinea pigs following

administration of sublethal doses of diphtheria toxin, suggest at least the possibility that recovery from poliomyelitis may be accompanied with a mobilization of vitamin C. Of particular interest is the fact that unusually high C levels were uniformly encountered in monkeys which had escaped paralysis following treatment with vitamin C, when such animals were examined in the early stages of their survival. Since these values were even higher than those obtained in normal animals after C preparation, they probably represent the aggregate effect of increased intake and increased assimilation of vitamin C during the infection. The artificial character of this high C saturation is clearly indicated by its brief duration, there being a return to normal levels in non-paralytic survivors which had not been examined until 6 weeks following the date of infection. Comparable figures have only been found in a small number of monkeys, not included in this work, which had passed through a non-paralytic febrile episode following intracerebral injection of poliomyelitis virus in combination with certain inactivating agents. It would seem likely therefore that the therapeutic effect observed after parenteral administration of vitamin C consists essentially in changing the frank paralytic type of the disease into the non-paralytic abortive type which fails to induce specific immunity to reinfection (11).

In conclusion it can be stated that distinctly hypernormal C levels in the central nervous system and in the suprarenals have been observed only in association with resistance to the disease. The possibility suggests itself, therefore, that there may be a certain delimiting threshold concentration of vitamin C in the tissues above which the monkey is protected and below which it is unprotected against the production of nerve cell damages by the virus. As this concentration begins to drop following discontinuance of C administration there is evidently a return to the state of susceptibility as indicated by the considerable number of animals which develop delayed paralysis between 14 days and 47 days following infection (12). We have no explanation to offer why only a certain percentage of infected monkeys following C administration are capable of reaching a critical concentration of vitamin C in the tissues and succeed in escaping paralysis permanently. But our data suggest that the success or failure of C treatment in experimental poliomyelitis may be intimately

connected with the extent to which the ascorbic acid is utilized by the organism.

SUMMARY AND CONCLUSIONS

1. The concentrations of reduced ascorbic acid present in the tissues of normal *rhesus* monkeys are of a magnitude in keeping with the values found for other animal species which are incapable of synthesizing vitamin C. These concentrations are subject to distinct increase by prolonged parenteral administration of ascorbic acid.

2. The amounts of vitamin C present in nervous tissue and the suprarenals of monkeys, paralyzed as the result of poliomyelitis infection, are slightly below the normal average when examined at the height of paralysis or in early convalescence. The figures show a tendency for a return to normal or slightly hypernormal levels concomitant with the progress of convalescence.

3. Vitamin C titrations of the tissues of monkeys which had received parenteral injections of ascorbic acid during the incubation period of poliomyelitic infection give different results according to whether such animals develop paralysis or survive without paralytic symptoms. In paralyzed C-treated monkeys the vitamin C levels are practically identical with those of normal C-prepared monkeys. Markedly higher values, however, are obtained with non-paralytic survivors in the early stages of their survival. As the period of survival lengthens normal figures prevail again.

4. The data are discussed in their relationship to the success or failure of vitamin C therapy in experimental poliomyelitis.

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THE INFLUENCE OF PROLONGED, INTENSIVE PLASMA-PHERESIS UPON THE ABILITY OF THE ORGANISM TO REGENERATE SERUM PROTEIN*

By DANIEL MELNICK,** Ph.D., and GEORGE R. COWGILL, Ph.D.

(*From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*)

The serum protein concentration has frequently been observed to be reduced in certain types of Bright's disease and in various types of malnutrition. Formerly the fundamental cause for this was attributed to either a loss of protein in the urine or a lack of protein in the diet. In 1933 Bloomfield (1), in introducing his studies on the effect of restriction of protein intake on the serum protein concentration of the rat, critically examined the evidence in support of the loss and lack theory and concluded that such a simple concept did not fit all the facts of clinical and experimental experience. The hypothesis was advanced that some other factor, such as an impairment of or an injury to the serum protein-regenerating mechanism, may be an accessory, if not the primary, factor in the production of a hypoproteinemia. More recent investigations, reviewed elsewhere (2), afford further support for this hypothesis.

In one of our previous papers (3) and in the communication coming from Whipple's laboratory (4) it was reported that the dog subjected to prolonged plasmapheresis may develop some impairment in ability to regenerate serum protein. In both of these studies the animals subsisted during alternate periods on adequate diets on the one hand and on low protein rations on the other. In our current studies we have been interested in determining as precisely as possible the factor responsible for the inhibition of serum protein formation; namely,

whether prolonged intensive loss of protein from the body *per se* is the etiologic agent or whether lack of protein is also a contributing factor. In the present investigation we have been concerned simply with the first phase of this problem. For this purpose the animals were fed adequate casein rations during the long régime of intensive plasmapheresis. Such a procedure, we believe, should also indicate whether or not prolonged proteinuria *per se* in nephrotic individuals, subsisting on adequate diets, predisposes to an impairment in ability to regenerate serum protein.

Methods

Plasmapheresis, the repeated bleedings of an animal, followed immediately by the reinjection of the corpuscular elements of the blood suspended in Locke's solution, was carried out according to the quantitative technique described in our previous publication (5). For the determination of the serum protein concentration both a nephelometric procedure (6) and a gravimetric method (7, 8) were employed. In estimating the blood volume use was made of the dye method as improved by Hooper, Smith, Belt and Whipple (9). Inasmuch as stasis which obstructs the venous return may cause an appreciable increase in the serum protein concentration (10), samples for analysis were taken either from the femoral artery or from the cubital vein under minimal stasis.

Recently Plötner (11, 12) has reported that variations in the albumin to globulin ratio in serum markedly affected the accuracy of his particular nephelometric procedure for determining the concentration of total serum protein. With a decrease in the ratio the observed turbidities were less than the corresponding values determined gravimetrically. The hypoproteinemia produced experimentally by the technique of plasmapheresis has been reported to be associated with a decreased or inverted albumin to globulin ratio (13–20, 4). The dogs used in the present study have also shown such changes in this ratio.[1] In our current investigations more than 100 cross determinations with both the nephelometric procedure (6) and the very accurate gravimetric method (7, 8) were conducted with the sera obtained from our dogs. The results of these estimations have indicated that there is a tendency, when the albumin to globulin ratio is inverted, for the nephelometric values to be less than those obtained by the standard gravimetric procedure. At times the difference may actually be as much as 15 per cent of the true value. The validity of the albumin to globulin ratio as determined in the clinic has been discussed elsewhere (21). We are of the opinion that such protein fractions are simply artificial products produced by the reagents employed in their preparation; that the serum protein complex is composed of

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[1] The authors express their gratitude to Dr. Daniel C. Darrow of the Department of Pediatrics, Yale University, for these determinations.

two or more unstable coprecipitation systems in mutual equilibrium; and that the particular protein fractions obtained indicate merely the potentialities of certain components in the serum to combine, probably by means of residual valencies, into a product that is insoluble under the imposed conditions. In our present studies we have attempted to eliminate any differences in the degree of dissociation of these reversible systems or in the tendency to dissociate which are believed to be responsible for the particular albumin to globulin ratios obtained. We have found that heating the aliquot of the diluted serum at 90°C. until the first faint turbidity is manifest (from 1 to 3 minutes), cooling and subjecting this to the subsequent steps in the nephelometric procedure will cause no appreciable change in the relative turbidities of normal sera samples when comparisons are made with the standard treated in the same manner. However, with sera, in which the albumin to globulin ratio is inverted, a significant change in the relative turbidities occurs so that better agreement with the gravimetric results is recorded. With this modification in the technique the average percentage deviation of the nephelometric values from the gravimetric was found to be approximately ±3.0 per cent.

This nephelometric procedure was used merely to calculate each day the volume of blood to be withdrawn in the plasmapheresis, according to the method described elsewhere (5). Twice each week these determinations were checked against the values recorded by the gravimetric method. For the important determination of the total amount of serum protein removed each week in order to maintain the blood protein concentration at the basal level, the gravimetric procedure was always used, after removal of the fibrin from the pooled citrated plasmas (22).

EXPERIMENTS

In the present study the initial experiments dealt with the standardization of the dogs so that, if any impairment in serum protein formation should occur, it would be possible to determine both qualitatively and quantitatively just what phase was affected, the endogenous or exogenous. The determination of the protein minimum for nitrogen equilibrium with each of the animals has been shown on theoretical grounds to be a prerequisite for the proper evaluation of that particular dog's ability to regenerate serum protein (3).

Three adult female dogs were used. At an optimal nutritive index of 0.30 (23), dog 4-6, a hound, weighed 14.2 kilos; dog 5-1, an airedale, 14.8 kilos, and dog 5-2, also a hound, 15.9 kilos. Preliminary to the nitrogen balance studies the animals were given a vermifuge and then offered an excess of vitamin B (B₁) to saturate the tissues with this dietary essential.[2] The dogs were then fed an

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[2] As a source of vitamin B (B₁) during these preliminary periods the dogs were fed a preparation called embo, powdered wheat embryo; this was kindly furnished by the General Mills, Inc., Minneapolis.

artificial "synthetic" diet, containing casein as the sole source of protein and fed at a level of 8 per cent of the caloric intake. The plan of the ration followed the kilo-unit scheme (24) and is similar to those used in our previous studies (22, 3, 25). The sample of casein used throughout the present investigation was a commercial preparation,[3] which contained 13.08 per cent nitrogen. This was indicative of an 82.3 per cent purity (26). The vitamin adjuvants[4] were identical to those employed in our previous studies (22, 3, 25) and were administered in the same amounts apart from the diet. These supplements are practically free of protein and therefore did not introduce any appreciable amount of that foodstuff into the diet. The animals were fed the ration each day at the maintenance level of 70 calories per kilo of optimal body weight; the vitamin adjuvants yielded an additional 1.5 calories per kilo.

In estimating the casein minimum for nitrogen equilibrium we have followed the method outlined in detail elsewhere (25). Table I summarizes the experimental findings. Calculations indicated that the protein minima for dogs 4-6, 5-1 and 5-2, expressed in terms of total ingested calories furnished by the protein in the diet, were 9.8, 8.55 and 9.45 per cent, respectively.

For the biological assay of the ability of each of these animals to regenerate serum protein, "synthetic" artificial diets were constructed similar to that used in the simple nitrogen balance studies but containing a casein increment of 0.75 gm. of the pure protein per kilo-unit, over and above the casein minimum required normally for each of the dogs. The protein content of the ration was increased at the expense of an isodynamic portion of the carbohydrate fraction, the other constituents remaining constant.

Subsequent to the nitrogen balance studies dogs 4-6, 5-1 and 5-2 were subjected to a standardized plasmapheresis régime, described in detail elsewhere (22), in order to evaluate their respective abilities to regenerate serum protein. Detailed calculations of the potency ratios are given below. The results indicate that these animals were normal at the beginning of this study with respect to their abilities to regenerate serum protein from materials obtained from both exogenous and endogenous sources.

Dog 4-6.

65 gm. casein = increment above the minimum for nitrogen equilibrium of 170 gm.

40.8 gm. serum protein were regenerated during the equilibrium week.

9.8 gm. serum protein = endogenous serum protein regeneration, animal subsisting on the protein-free diet (22).

Therefore,

$$\frac{40.8 - 9.8}{65} = 0.48, \text{ potency ratio.}$$

Dog 5-1.

68 gm. casein = increment above the minimum for nitrogen equilibrium of 155 gm.

33.4 gm. serum protein were regenerated during the equilibrium week.

5.7 gm. serum protein = endogenous serum protein regeneration, animal subsisting on the protein-free diet (22).

Therefore,

$$\frac{33.4 - 5.7}{68} = 0.41, \text{ potency ratio.}$$

Dog 5-2.

73 gm. casein = increment above the minimum for nitrogen equilibrium of 185 gm.

46.2 gm. serum protein were regenerated during the equilibrium week.

9.6 gm. serum protein = endogenous serum protein regeneration, animal subsisting on the protein-free diet (22).

Therefore,

$$\frac{46.8 - 9.6}{73} = 0.51, \text{ potency ratio.}$$

According to our experimental plan the animals were maintained on the adequate casein rations and were subjected to quantitative plasmapheresis for a total of 12 weeks. Following this the protein diet was replaced by the protein-free ration in order to determine whether any impairment in ability to utilize the products of tissue protein catabolism had developed as a consequence of the prolonged, intensive plasmapheresis. The results of these studies, including the periods which furnished the data used in the biological evaluation of casein with respect to serum protein regeneration, are presented in Tables II, III and IV. The details of plasmapheresis are summarized for each period of 7 days. The variations in body weight, in blood and plasma volume and in the hematocrit are also included in these tables, because (a) they afford some indication of the nutritional status of the animals and (b) they constitute a kind of index of the composition of the blood during such a long experimental régime.

When these experiments were begun the weights of the dogs approximated those associated with an optimal nutritive condition,

as estimated by the nutritive index formula (23). Throughout the study all three animals evidenced a progressive increase in body weight so that the final values were approximately from 14 to 19 per cent greater than the initial optimal weights. Barker and Kirk (27) have reported a decreased basal metabolism in dogs subjected to plasmapheresis. Clinically, in patients with the condition known as nephrosis, a similar decrease of from 20 to 30 per cent is frequently

TABLE I

Estimation of the Casein Minimum for Nitrogen Equilibrium

Dog No.	Experimental period	Basal ration* per day		Vitamins† per day		N intake per day		N excretion per day		Average weight of dog	Period; N balance	Casein minimum‡
						Basal	Vitamins	Urine	Feces			
	days	gm.	calories	gm.	calories	gm.	gm.	gm.	gm.	kg.		percent calories
4-6	6	202	994	5.25	21	3.149	0.251	—	—	14.42	Adjustment	9.8
	4	202	994	5.25	21	3.149	0.251	3.380	0.451	14.35	−0.431 gm. N	
5-1	6	211	1036	5.5	22	3.290	0.262	—	—	14.29	Adjustment	8.55
	4	211	1036	5.5	22	3.290	0.262	3.188	0.500	14.46	−0.136 gm. N	
5-2	6	227	1113	6.0	24	3.539	0.281	—	—	15.32	Adjustment	9.45
	4	227	1113	6.0	24	3.539	0.281	3.675	0.545	15.36	−0.400 gm. N	

* The plan of this ration followed the kilo-unit scheme (24); the diet furnished 8 per cent of the caloric intake in the form of protein and was fed at the maintenance level of 70 calories per kilo of optimal body weight as estimated by the nutritive index formula (23).

† The vitamin adjuvants and the quantities administered are described in a previous publication (22 or 25).

‡ The protein minima for these animals were estimated from the nitrogen balance data by the method reported in detail elsewhere (25).

manifest (28). Thus, the increases in body weight in our dogs, subsisting on a diet furnishing calories in amount normally adequate just for the maintenance requirements, are probably due to an appreciable extent to a decrease in their basal metabolic rates.

In these experiments, as in the case of our earlier studies (22, 3), a reduction in the serum protein concentration produced by plasmapheresis is associated with a drop in the blood volume. However,

when there is also a significant reduction in the cell volume leading to a passive reduction in the blood volume, then the lowered osmotic

TABLE II

The Influence of Prolonged, Intensive Plasmapheresis upon the Ability of Dog 4-6 to Regenerate Serum Protein

Optimal weight* of dog = 14.2 kilos.

Diet†	Period (7 days)	Average weight of dog	Blood volume	Plasma volume	Average hematocrit	Total bleeding	Cell exchange		Citrated plasma			Serum protein removed per week
							Bled	Injected	Citrate	Plasma	SP‡	
		kg.	*cc.*	*cc.*	*per cent*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*	*gm.*
Protein-free	I	14.92	1237	650	46.5	1853	860	838	204	993	4.80	47.7
	II	14.64	1150	605	41.2	415	174	245	46	241	4.04	9.8
Casein increment	III	14.76	1026	613	38.8	988	384	508	110	604	4.23	25.6
	IV	15.24	1057	658	40.2	1487	594	561	165	893	4.57	40.8
	V	15.48	1003	592	39.8	1453	570	626	161	883	4.43	39.2
	VI	15.70	1110	636	39.9	1446	580	654	161	866	4.37	37.8
	VII	15.94	1040	618	41.7	1366	566	622	152	800	4.45	35.6
	VIII	16.21	1172	671	39.0	1248	486	452	137	762	4.41	33.6
	IX	16.46	1065	661	42.1	1122	473	576	119	649	4.50	29.2
	X	16.67	1086	644	43.6	1390	604	650	155	786	4.55	35.8
	XI	16.97	1154	637	40.7	1328	539	530	147	789	4.57	36.0
	XII	16.96	1007	593	43.0	1415	610	688	158	805	4.66	37.6
	XIII	17.33	1074	610	41.6	1562	655	634	170	907	4.54	41.2
	XIV	17.25	1121	671	44.6	1490	659	710	165	831	4.61	38.3
Protein-free	XV	16.92	1150	639	43.0	1104	476	492	124	628	4.35	27.3
	XVI	16.93	1138	658	41.0	459	193	213	51	266	4.12	11.0

* Calculated for the animal when adjusted to an optimal nutritive condition, as estimated by the nutritive index formula (23).

† The protein-free diet was fed daily at a 90 calorie per kilo level. The casein increment ration was administered daily, 70 calories per kilo of optimal body weight. The vitamin adjuvants furnished in addition 1.5 calories per kilo of body weight.

‡ The symbol SP represents the concentration of serum protein in the pooled plasma aliquots after corrections had been made for the presence of the citrate and calcium chloride solutions.

effect due to a reduced serum protein concentration appears to be negligible in effecting a further reduction in the blood volume (29). Under such conditions the plasma volume may actually be greater

than the normal.   Such has frequently been the case in the present study with dogs 4-6, 5-1 and 5-2.

Throughout the current investigation the hematocrit values were always below the initial readings but were kept well within the normal range.   When dogs are subjected to plasmapheresis, the

TABLE III

The Influence of Prolonged, Intensive Plasmapheresis upon the Ability of Dog 5-1 to Regenerate Serum Protein

Optimal weight * of dog = 14.8 kilos.

Diet*	Period (7 days)	Average weight of dog	Blood volume	Plasma volume	Average hematocrit	Total bleeding	Cell exchange		Citrated plasma			Serum protein removed per week
							Bled	Injected	Citrate	Plasma	SP*	
		kg.	*cc.*	*cc.*	*per cent*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*	*gm.*
Protein-free	I	15.13	1320	653	47.6	1517	726	742	170	791	4.86	38.4
	II	15.20	1131	616	44.3	249	111	110	28	138	4.11	5.7
Casein increment	III	15.09	1114	620	44.3	559	248	271	61	311	4.17	13.0
	IV	15.09	1121	628	45.0	1297	583	574	144	714	4.68	33.4
	V	15.48	1215	656	42.3	1327	562	559	148	765	4.62	35.4
	VI	15.75	1160	672	45.8	1334	613	665	147	721	4.56	32.9
	VII	16.00	1198	633	44.0	1261	555	577	140	706	4.43	31.3
	VIII	16.27	1110	640	43.8	1350	591	580	150	759	4.60	34.9
	IX	16.44	1157	658	41.7	1293	541	540	145	752	4.27	32.2
	X	16.61	1120	665	42.2	1154	485	523	127	669	4.54	30.4
	XI	16.48	1082	620	41.8	1265	525	524	140	740	4.35	32.2
	XII	16.61	1111	625	41.9	1195	501	518	131	694	4.36	30.2
	XIII	16.78	1090	630	41.2	1260	520	517	141	740	4.46	33.0
	XIV	16.75	1077	627	42.8	1231	524	567	138	707	4.42	31.3
Protein-free	XV	16.79	1160	657	43.2	663	281	270	74	382	4.11	15.7
	XVI	16.81	1056	572	42.1	206	87	78	23	119	3.96	4.7

* The same qualifying statements, added as footnotes to Table II, apply also to this table.

hematocrit tends to approach anemia levels unless donor cells are injected periodically.   In our previous studies (5) the cells were separated from the citrated plasma, subsequent to each bleeding, and taken up in physiological saline.   It was in such a solution that the cells were stored in the refrigerator until the next plasmapheresis.

For the reinjection of these cells the saline was replaced by an approximately equivalent volume of modified Locke's solution (30). In the present study the latter was altered by the addition of 2.5 gm. of glucose per liter of solution. Cells were always stored for further use and always reinjected, suspended in this modified

TABLE IV

The Influence of Prolonged, Intensive Plasmapheresis upon the Ability of Dog 5-2 to Regenerate Serum Protein

Optimal weight* of dog = 15.9 kilos.

Diet*	Period (7 days)	Average weight of dog	Blood volume	Plasma volume	Average hematocrit	Total bleeding	Cell exchange		Citrated plasma			Serum protein removed per week
							Bled	Injected	Citrate	Plasma	SP*	
		kg.	*cc.*	*cc.*	*per cent*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*	*gm.*
Protein-free	I	15.81	1341	667	49.5	1879	929	931	208	950	4.88	46.4
	II	15.72	1268	640	46.5	456	213	280	50	243	3.95	9.5
Casein increment	III	15.79	1188	638	43.8	915	400	480	102	515	4.18	21.5
	IV	16.29	1205	705	39.3	1666	657	645	185	1009	4 64	46.8
	V	16 63	1175	726	40.1	1769	708	768	197	1061	4.47	47.5
	VI	16 95	1201	705	40.6	1755	714	823	195	1041	4.50	46.8
	VII	17.06	1197	725	39.3	1750	692	732	194	1058	4.55	48.1
	VIII	17.43	1176	710	39.6	1819	722	783	202	1097	4.52	49.6
	IX	17.61	1198	765	38.2	1702	649	880	189	1053	4.76	50.1
	X	17.94	1198	728	38.5	1874	722	935	209	1152	4.79	55.1
	XI	18.11	1253	716	38.9	1787	692	928	198	1095	4.71	51.6
	XII	18.30	1223	710	41.1	1683	690	798	188	993	4.78	47.5
	XIII	18 57	1170	680	41.8	1804	754	752	200	1050	4.60	48.3
	XIV	18.75	1264	732	44 7	1925	862	944	213	1063	4.51	47.9
Protein-free	XV	18.90	1169	660	43.5	1320	573	614	147	747	4.52	33.8
	XVI	18.78	1235	685	43.3	518	235	181	58	283	4.18	11.9

* The same qualifying statements, added as footnotes to Table II, apply also to this table.

solution. We are of the opinion that the addition of the glucose exerts a profound effect upon the stability of the red blood cells. Thus in our previous investigations (22, 3) an excess of red blood cells amounting to actually 15 per cent of the volume removed had to be reinjected in order to maintain a normal hematocrit; in our

present study an excess of only 5.5 per cent was required, despite the fact that in these experiments the animals were subjected to prolonged plasmapheresis. For some unknown reason in the case of dog 4 during periods IX and X, described in the previous paper (3), and in the case of dog 5-2 during periods IX, X and XI in the present study there was a marked destruction of the red blood cells *in vivo*. This necessitated the injection of a huge excess of red blood cells in both cases of approximately 33 per cent more than that withdrawn. The amount of red cell destruction during these periods was not averaged in determining the relative degrees of hemolysis in both studies, indicated above. Of particular interest is the fact that during the periods of increased red blood cell destruction *in vivo* both dogs exhibited significant increases in the potency ratios when compared with similar periods but in which the destruction of the erythrocytes were not nearly so great. For this reason we believe that the hemoglobin thrown into the circulation by the hemolysis of the injected red cells is broken down and serves as an additional source of amino acids for promoting the regeneration of serum protein. It is our opinion that this "globin variable" exhibits not only an additive but also a supplementary effect upon the dietary protein, so that the results obtained in our studies as well as in those reported by others cannot be referred solely to the dietary protein.

In the current investigation we have been interested primarily in the ability of the organism to regenerate serum protein when exposed to a régime of prolonged, intensive plasmapheresis. During the initial depletion period and the subsequent equilibrium week the dogs subsisted on the high caloric protein-free diet. This ration was then replaced by the casein increment diets, the animals still being subjected to quantitative plasmapheresis. After the adjustment period equilibrium values were obtained and these were used in arriving at the potency ratios calculated above. Subsequently dog 4-6 showed a slow but progressive decrease in ability to regenerate serum protein, but this proved to be only temporary in nature, since it was followed by a prompt rise until the initial equilibrium value was again attained. Dog 5-1 throughout the corresponding periods evidenced no significant changes in its capacity to produce this blood protein. Dog 5-2 behaved in a similar manner. The significant

increase in serum protein output recorded for periods IX, X and XI bas been attributed to the considerable hemolysis which occurred during these weeks. Thus, in all three dogs we have observed no notable impairment in ability to regenerate serum protein. Evidently these animals, if our hypothesis (3) is true, should still be able to utilize adequately the products of tissue protein catabolism (endogenous serum protein regeneration) for the formation of this blood protein. This was confirmed by changing the animals' rations back to the protein-free diet, the régime of quantitative plasmapheresis still being maintained. After the depletion periods, the serum protein outputs were approximately equal to those recorded for the comparable equilibrium weeks at the beginning of these experiments.

DISCUSSION

In our previous paper (3), reporting an impairment of the ability of dog 3 to regenerate serum protein, this animal had been subjected to a total of 15 weeks of plasmapheresis, subsequent to a rest period of 4 months. As was mentioned earlier in this paper the animal subsisted during alternate periods of 2 weeks on the protein-free diet on the one hand and on the adequate protein rations on the other. In other words this animal was subjected at times to both a loss of protein by bleeding and a lack of protein from the diet. In the present study only the "loss" factor was made to operate. Evidently prolonged plasmapheresis *per se*, resulting in the formation and loss of large amounts of serum protein, does not cause an impairment in the ability of the organism to regenerate this blood protein.

Peters and his associates (31) point out that in conditions of nephritic edema associated with marked proteinuria the patients frequently are malnourished due specifically to protein starvation. The evidence for such a conclusion was based on the prolonged positive nitrogen balances recorded for these individuals when they subsisted on diets containing high calories and more than maintenance quantities of protein. In our experiments with the dog, rations furnishing approximately 40 per cent of protein above the maintenance requirements were fed. Under such conditions in spite of marked losses of serum protein the animals suffered no disturbance in ability to regenerate this complex. Peters (31) emphasizes that besides

proteinuria the chief cause of the serum protein deficiency appears to be depletion of the protein stores of the body. In the present study it seems that we have ruled out protein loss as being the significant factor in causing an impairment in serum protein formation. Experiments are planned for the future in which the dogs will be subjected to the protein-free diet (lack of protein) in addition to quantitative plasmapheresis (loss of protein); under such conditions we feel that the change in ration to the adequate casein diet will result in a greatly decreased output of serum protein when compared with the previous control periods.

It may occur to the reader that a period of uninterrupted plasmapheresis of 16 weeks duration is possibly too short to warrant any significant conclusions. For this reason we have attempted to equate the present findings recorded with the dog with those theoretically possible with the human species. In our studies seven dogs have been standardized with respect to their ability to regenerate serum protein, while subsisting on adequate casein rations. The results obtained with dogs 3 and 4 have been described in a previous publication (3); the experiments with dogs 4-6, 5-1 and 5-2 were discussed in the present report. Dogs 4-7 and 4-8 were standardized for use in another study; a brief summary of these tests including those conducted with the other animals is presented in Table V.

Consideration of the data present in this table indicates that these dogs, fed diets containing casein at a level of about 13 per cent of the caloric intake, can regenerate each week approximately 93 per cent of the amount of serum protein initially present in the plasma. The quantity of protein fed to the dogs was liberal in so far as satisfying the minimal nitrogen requirements were concerned, but the rations used are not to be considered as being so called "high protein" diets. Thus, in the case of a 70 kilo man under similar environmental conditions this would be equivalent to the individual receiving only about 80 gm. of protein per day. The normal life span of the human being is approximately 4.5 times as long as that of the dog. A similar relationship is observed during pregnancy in both species with respect to the length of the normal gestation period. If such a ratio is accepted as being valid, then the 16 weeks of uninterrupted plasmapheresis conducted in the present study may be considered

TABLE V

Serum Protein Regeneration in Dogs Fed an Adequate Diet in Which Casein Was the Sole Source of Protein and Was Fed at a Level of Approximately 13 Per Cent of the Caloric Intake

Dog No.	Breed and sex	Optimal weight*	Protein minimum for nitrogen equilibrium	Casein increment fed per week†	Initial total circulating serum protein	Total serum protein regeneration per week	Endogenous serum protein regeneration per week‡	Potency ratio§
		kg.	per cent calories	gm.	gm.	gm.	gm.	
3	Hound ♀	16.7	9.1	76	44.4	47.4	13.4	0.45
4	Collie ♀	17.3	8.9	92	52.1	48.6	9.6	0.42
4-6	Hound ♀	14.2	9.8	65	43.3	40.8	9.8	0.48
4-7	Terrier ♀	12.2	8.65	56	38.0	32.4	5.3	0.48
4-8	Terrier ♀	9.5	8.7	44	26.6	21.5	6.3	0.35
5-1	Airedale ♀	14.8	8.55	68	40.8	33.4	5.7	0.41
5-2	Hound ♀	15.9	9.45	73	46.2	46.8	9.6	0.51
Average values per kilo of body weight			2.9 (±0.15)(av.d.)		2.9 (±0.15)(av.d.)	2.7 (±0.2)(av.d.)	0.6 (±0.1)(av.d.)	0.44 (±0.4)(av.d.)

* These values are calculated for the animals when adjusted to an optimal nutritive condition as estimated by the nutritive index formula (23).

† This refers to the absolute amount of pure protein ingested each week over and above the minimum required for nitrogen equilibrium.

‡ By endogenous serum protein we refer to the ability of the dog under our experimental conditions to utilize the products of tissue protein catabolism for the formation of serum protein.

§ The potency ratio is expressed as the ratio of (a) the amount of serum protein per week removed by the bleedings above that regenerated from endogenous sources, to (b) the dietary protein increment, i.e. the amount above that required for nitrogen equilibrium.

as being equal to a period of 72 weeks in the case of man. In similar fashion the 12 weeks of intensive prolonged plasmapheresis, during which the animals subsisted on the adequate casein rations, may be regarded as equivalent to 54 weeks in the human species. According to Peters and Van Slyke (28) the normal average serum protein concentration in human beings is approximately 7.0 per cent and the average plasma volume (dye method) is about 54 cc. per kilo. Thus for an average 70 kilo man the plasma volume should total 3780 cc. and should contain 265 gm. of serum protein. Let us suppose that such an individual subsequently develops a marked proteinuria and hypoproteinemia, as a result of which he is actively regenerating serum protein. If the ability of the human being to form this blood protein is assumed to be equal to that of the dog, then calculations (265 gm. serum protein × 0.93) indicate that the 70 kilo man should be able to regenerate as much as 247 gm. of serum protein per week. In other words such an individual fed an adequate ration should be able to lose each day as much as 35 gm. of serum protein over a period of 54 weeks without suffering any impairment in ability to regenerate this blood protein.

The findings in the present study emphasize the remarkable ability of the normal organism to regenerate rapidly serum protein over a relatively long period of time. It seems to us that in clinical conditions of Bright's disease associated with a proteinuria in which there may be a daily loss for example of even 20 gm. of protein per day some other factor must be postulated for explaining the persistence of the hypoproteinemia. It seems obvious that, if loss of protein is to be effective in lowering the protein concentration, it must be extremely large unless there is in addition some impairment of a mechanism responsible for the formation of serum protein.

With respect to such a mechanism it is of interest to compare the ability of the dog to regenerate serum protein with its efficiency in using dietary protein for meeting the general nitrogen requirements. The data presented in Table V show that the respective potency ratios recorded for each of the dogs are independent of the protein minima values. This observation seems to indicate that the regeneration of serum protein bears no definite relation to the nitrogen metabolism of the organism as a whole but may be dependent

upon a specific mechanism responsible for the formation of the blood protein.

SUMMARY

1. When the dog is subjected to quantitative plasmapheresis and fed appropriate "synthetic" artificial rations, it is possible to evaluate the ability of the organism to regenerate serum protein from both exogenous and endogenous sources. Approximately 44 per cent of the protein, casein, fed in excess of the minimal amount needed normally to meet the general nitrogen requirements, is utilized for the formation of new serum protein. Under our experimental conditions the dog can regenerate each week solely from endogenous sources approximately 0.6 gm. of this blood protein per kilo of optimal body weight. This is equivalent to about 21 per cent of the total amount of serum protein normally present in the plasma.

2. When the dog is fed an adequate protein diet and is subjected to a régime of prolonged intensive plasmapheresis (period of 16 consecutive weeks), no impairment in the ability of the organism to regenerate serum protein from either exogenous or endogenous sources occurs. Under our conditions of experimentation the dog appears to be able to form each week an amount of the blood protein approximately equal to that *normally* present in the plasma. Because of this remarkable ability of the normal organism to regenerate rapidly serum protein over a relatively long period of time, it seems that loss of protein *alone* in conditions of Bright's disease cannot be the etiologic agent responsible for the persistence of the hypoproteinemia. An additional factor, the "specific" ability of such individuals to regenerate serum protein, must be taken into consideration.

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THE INFLUENCE OF PREGNANCY AND LACTATION UPON THE REGENERATION OF SERUM PROTEIN*

By DANIEL MELNICK,** Ph.D., and GEORGE R. COWGILL, Ph.D.

(From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven)

It has long been known that there are changes in the blood volume during pregnancy, and that these are associated with a hydremia due almost entirely to an increase in the water content of the plasma (1–6). Various fluctuations in the concentration of plasma proteins (7–12, 6) occur during all stages of pregnancy and they appear in large measure to be associated with this hydremia, although Peters and Van Slyke (14) have suggested that loss of blood during labor may play a rôle. Davis and Bodansky (13) have reported observations made on rabbits, a species which delivers large litters, from which it appears that the delivery of large litters intensifies the process by which the concentration of plasma proteins is lowered. They reported that the lowest concentration occurred 12 hours after parturition; the normal concentration was not attained during the 25 day period of lactation.

In the present investigation we have been interested primarily in the transitory changes in the serum protein concentration which take place during terminal pregnancy and early lactation. It seems to us that animals, which deliver relatively large litters of young, are better suited for such a study. Inasmuch as the "strain" of pregnancy and lactation in these species may be assumed to be greater than that in cases of human pregnancy, one might expect to obtain values which because of their distinct differences would be of greater significance. Such has been the case in the studies conducted

by Davis and Bodansky (13) on rabbits during pregnancy and lactation. Furthermore, we have been impressed with the relatively low serum protein concentrations recorded at parturition, which in the case of the rabbit may actually approximate the low values recorded for significant hypoproteinemias. In view of the remarkable ability of the normal organism to regenerate serum protein (15), the possibility suggests itself that in conditions of pregnancy and early lactation there may be a limited store of reserve serum protein or an impairment of the ability of the organism to form this blood protein or an involvement of both of these factors. Indeed, the synthesis of body proteins in the fetus during pregnancy and the milk proteins during lactation may be considered to be actually an internal plasmapheresis, leading to a depletion of the serum protein by the preferential utilization of the material from which this complex is made. In the present study by means of rigid control of the dietary intake and by use of the technique of quantitative plasmapheresis we have attempted to evaluate the ability of the organism to regenerate serum protein during pregnancy and lactation.

Methods and Material

The methods followed were practically the same as those used in our previous investigations of serum protein regeneration (16–18, 15). The concentrations of the serum protein were determined for the most part by the nephelometric procedure of Rona and Kleinmann (19), modified as described in our previous communication (15). Periodically, these values were checked by determining the concentration of the blood protein by the direct gravimetric procedure (20, 21), especially on those days when the serum protein concentration showed dramatic changes. The gravimetric method was always used for determining the concentration of the serum protein in the pooled plasma aliquots, after the fibrin had been removed according to the procedure described elsewhere (17). For the estimation of the blood volume, the dye method as improved by Hooper, Smith, Belt and Whipple (22) was employed. Because obstruction to the venous return very quickly increases the protein concentration in the plasma (23), the samples used for the determination of the serum protein concentration and blood volume were taken in most cases from the femoral artery. Since we were very much interested in the blood volume changes and did not want to affect them appreciably by our methods of analyses, the blood samples used for the various determinations were as small as possible. Thus, in the case of a serum protein estimation 2 to 3 cc. of blood were withdrawn, while in conducting a blood volume determination the animal lost a total of only 10 cc. of blood. Plasmapheresis, plasma depletion

effected by the repeated bleedings of the animal, followed immediately by the reinjection of the corpuscular elements of the blood suspended in Locke's solution, was conducted according to the standardized technique described in our previous publication (16).

In the present investigation three diets were employed, a natural stock diet, a "synthetic" artificial protein ration, and a "synthetic" artificial protein-free diet. The natural stock ration[1] consisted of a mixture of natural foods and was occasionally supplemented with meat scraps obtained from the New Haven Hospital. The "synthetic" artificial protein diet was the casein III ration described by Cowgill (24), which contains casein[2] as the sole source of protein, with exception that a modified Osborne and Mendel salt mixture (25) replaced that described in the original formula. In our laboratory dogs have subsisted on this diet for as long as 18 months and throughout this period appeared to have been in an excellent nutritive condition. The protein-free diet is described in detail elsewhere (17). The same vitamin supplements[3] and the same quantities as described in that paper (17) were administered to the dogs, when they subsisted on either of the two "synthetic" rations.

RESULTS

The initial investigations are concerned with the variations in the serum protein concentration, blood volume, hematocrit, and plasma volume in the normal dog subsisting on the adequate "synthetic" casein diet, and in another animal fed the same ration during terminal pregnancy and early lactation.

Dog 1-0, a fox-terrier with an optimal weight of 8.95 kilos, calculated for the animal when adjusted to an optimal nutritive condition (24), was used as the normal control dog in this study. Dog 5-6, a mongrel, was estimated to have the same optimal weight and was employed to investigate the effects of pregnancy and lactation upon the composition of the blood. Prior to the present experiments both animals were fed the natural stock diet *ad libitum*. Subsequently the casein ration was administered, also *ad libitum*, supplemented with the calculated amounts of the vitamin adjuvants. Dog 1-0 subsisted on the "synthetic" diet for a period of 8 weeks during which time it evidenced a slow but progressive gain in weight until the final value was 10.2 kilos. Dog 5-6 was fed the casein ration

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[1] Tioga dog food, formerly baloration, obtained from the Tioga Mills, Inc., Waverly, New York.

[2] Obtained from the Lister Bros., New York.

[3] We are indebted to the Health Products Corporation, Newark, and to The Eli Lilly and Company, Indianapolis, for furnishing us with generous supplies of their cod liver oil concentrate tablets and liver 343 powder, respectively.

5 days before term, subsisted on it for the first 2 weeks of lactation and finally was returned to the natural stock diet.   This animal weighed 9.2 kilos before the litter was cast, 7.0 kilos *post partum* and finally 8.1 kilos when the normal blood picture
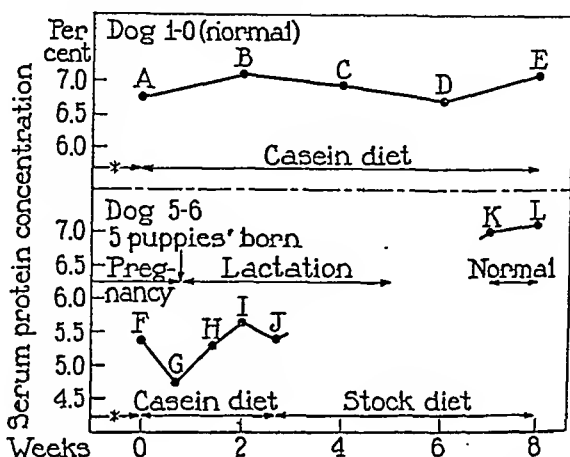
CHART 1. Variations in the serum protein concentration, blood volume, hematocrit and plasma volume in the normal dog, subsisting on an adequate "synthetic" casein diet, and in another animal fed the same ration during terminal pregnancy and early lactation.

* During these preliminary periods the dogs subsisted on a natural stock diet.

Dog 1-0					
Symbol.	A	B	C	D	E
Blood volume, cc.	807	823	852	807	780
Hematocrit, per cent.	43.5	42.7	42.4	43.6	44.5
Plasma volume, cc.	456	472	491	456	433

Dog 5-6						
Symbol.	F	G	H	I	J	K	L
Blood volume, cc.	746	746	677	680	679	757	747
Hematocrit, per cent.	31.5	31.1	32.7	33.7	35.7	49.0	50.5
Plasma volume, cc.	511	514	456	451	437	386	370

was again attained.   The effects of such a dietary régime upon the composition of the blood are presented in Chart 1.

Examination of the chart clearly indicates that the casein ration, when fed to the normal non-gravid dog, is adequate for the main-

tenance of a normal serum protein concentration and a normal blood and plasma volume. In the case of the pregnant animal there was a marked plasma hydration but no increase in the total blood volume, due to the fact that the hematocrit was extremely low. In human beings there is also a reduction of the hemoglobin concentration, cell count and cell volume somewhat too large to be explained simply by the hydremia (14). However, in the case of dog 5-6 the total cell volume showed actually a maximal decrease of approximately 40 per cent. As in the studies by Davis and Bodansky (13) of the composition of blood in rabbits during pregnancy and lactation, marked changes in the serum protein concentration were also recorded for this dog. Thus, the concentration of the blood protein 5 days before term was three-fourths and at parturition actually two-thirds of the normal value. However, when expressed as *total* circulating serum protein, there was no appreciable decrease except at parturition and early lactation, and then these values were only about 10 per cent less than normal. With human beings the changes in the serum protein concentration are not nearly as great and with respect to the total amount of serum protein there is generally an *increase* during pregnancy and a return to normal during the 1st week *post partum* (3, 6). The findings recorded with dog 5-6 indicate that the dog as well as the rabbit undergoes a considerably greater physiological strain during pregnancy and lactation than the human being. In this connection it is pertinent to point out that despite the fact that dog 5-6 subsisted on a diet which contains approximately 34 per cent protein it evidenced a significant hypoproteinemia during the periods of pregnancy and lactation which were under observation. This becomes even more significant when one considers that the normal dog subsisting on a ration which contains only from 15 to 16 per cent of the very same protein exhibits a remarkable ability to regenerate serum protein (18, 15). In the dog the duration of pregnancy is usually 63 days; the puppies are generally weaned after 42 days. In view of the markedly short period of gestation in this species and in view of the relatively large litters that are cast and nursed, the dog appears to be ideally suited for the experiments devised to evaluate the effects of normal pregnancy and lactation upon the ability of the organism to form serum protein.

TABLE I

The Amount of Reserve Serum Protein in the Normal Dog and the Ability of the Organism to Utilize the Products of Tissue Protein Catabolism for the Formation of Serum Protein

Dog No.	Breed and sex	Optimal weight*	SP removed†		Initial total circulating SP	Reserve SP stores‡	Endogenous SP regeneration per week‡
			Depletion week	Equilibrium week			
		kg.	gm.	gm.	gm.	per cent	per cent
3	Hound ♀	16.7	49.2	13.4	44.4	40	30
4	Collie ♀	17.3	56.4	9.6	52.1	52	19
4-3	Mongrel ♀	12.6	38.4	7.6	36.9	39	21
4-4	Fox-terrier ♀	10.0	38.0	9.2	37.2	36	25
4-6	Hound ♀	14.2	47.7	9.8	43.3	45	23
4-7	Terrier ♀	12.2	31.6	5.3	38.0	31	14
4-8	Terrier ♀	9.5	23.3	6.3	26.6	26	24
5-1	Airedale ♀	14.8	38.4	5.7	40.8	37	14
5-2	Hound ♀	15.9	46.4	9.6	46.2	35	21
Average values per kilo of body weight........			$3.0 \left(\pm 0.3 \atop \text{av.d.} \right)$	$0.62 \left(\pm 0.1 \atop \text{av.d.} \right)$	$3.0 \left(\pm 0.2 \atop \text{av.d.} \right)$	$38 \left(\pm 5.4 \atop \text{av.d.} \right)$	$21 \left(\pm 3.8 \atop \text{av.d.} \right)$

* These values are calculated for the animals when adjusted to an optimal nutritive condition as estimated by the nutritive index formula (24).

† The symbol SP is used as an abbreviation for serum protein. During these periods the dogs subsisted on the protein-free ration and were subjected to the standardized plasmapheresis procedure described in the text.

‡ Expressed in terms of the per cent of the total amount of circulating serum protein normally present. The actual amount of reserve serum protein is calculated by subtracting from the total amount of the blood protein removed during the initial depletion week both that amount required simply to reduce the serum protein concentration from the normal to the basal level and that amount which the animal regenerates when fed the protein-free diet during the equilibrium week. By endogenous SP regeneration we refer to the ability of the dog under our experimental conditions to utilize the products of tissue protein catabolism for the formation of serum protein.

Previous studies (17, 18, 15) have given some index of the amount of reserve serum protein, stored as such or potentially so, in the normal dog and the ability of such an organism to utilize the products of tissue protein catabolism for the formation of this blood protein. By the administration of the protein-free diet at a high level of caloric intake to the dog subjected to plasmapheresis during which one-fourth of the blood volume of the animal is withdrawn daily, it is possible to reduce the serum protein concentration to the basal level (3.5 to 4.2 per cent) and to deplete the organism of its reserve stores of this protein within 1 week. The subsequent week was demonstrated to be an equilibrium period, indicative of the amount of serum protein that can be regenerated with no protein in the diet and with tissue protein catabolism alone furnishing the materials from which the protein complex is made. Plasmapheresis during this latter period is performed quantitatively (16); whenever the serum protein concentration rises to 4.2 per cent or above, a calculated volume of blood is removed to reduce the level to 3.5 per cent. The results, obtained to date with nine dogs, are summarized in Table I, and indicate that the normal dog possesses a considerable reserve store of serum protein of approximately 38 per cent of the total amount normally present in the circulation. When fed the protein-free diet and when subjected to quantitative plasmapheresis the dog is able in 1 week to regenerate approximately 21 per cent of the total amount of the blood protein normally present in the plasma.

Dogs 4-3 and 4-4 were employed in the experiments devised to evaluate the effects of pregnancy and lactation upon the ability of the dog to regenerate serum protein. In order to present more clearly and in greater detail the normal control findings with these dogs, reference is made to Chart 2. The intensity and frequency of the plasmapheresis necessary to deplete these animals of their reserve serum protein stores, reduce their blood protein concentrations to the basal level and to maintain them there are indicated in the chart. The normal serum protein concentrations, blood and plasma volumes and hematocrit readings are presented, as well as the corresponding values when the dogs were subjected to plasmapheresis.

Both dogs consumed the "synthetic" artificial protein-free diet at the high level of caloric intake of 90 calories per kilo of optimal body weight. Dogs maintained in metabolism cages under similar environmental conditions but fed an adequate diet usually require

in the neighborhood of 70 calories per kilo for maintenance. Dog 4-3 during this experimental period evidenced a slow but progressive loss
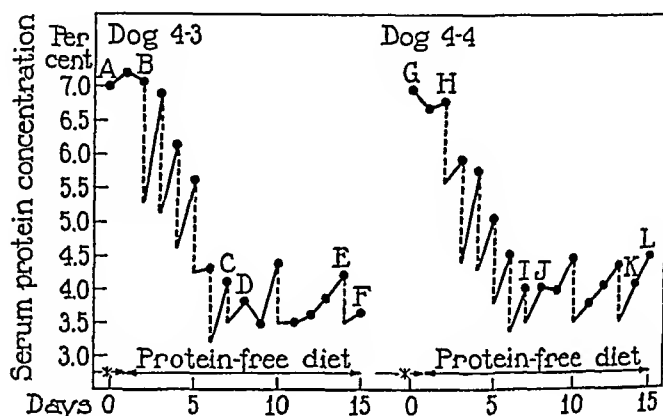
CHART 2. Serum protein depletion in the normal dog and the ability of the normal organism to utilize the products of tissue protein catabolism for the formation of serum protein. The dotted lines represent plasmapheresis.

* During this preliminary period the dogs subsisted on a natural stock diet followed by 1 week on an artificial "synthetic" casein ration.

Symbol	Blood volume	Hematocrit	Plasma volume	Period	Total bleeding		Serum protein removed
					Blood	Plasma	
Dog 4-3							
	cc.	per cent	cc.		cc.	cc.	gm.
A	1126	53.8	520	B–D	1577	773	38.4
C	942	48.0	490	D–F	343	186	7.6
E	965	44.4	537				
Dog 4-4							
G	957	44.0	536	H–J	1322	748	38.0
I	890	38.4	548	J–L	374	226	9.2
K	846	41.8	492				

of body weight from 13.7 to 12.7 kilos; dog 4-4, from 10.6 to 10.2 kilos. The optimal body weights of these animals were calculated to be 12.6 and 10.0 kilos, respectively.

The influence of pregnancy and lactation upon the ability of these dogs to regenerate serum protein is presented in Charts 3 and 4.

In these experiments the animals were fed initially the natural stock diet *ad libitum* followed by the "synthetic" casein diet, also *ad libitum*. This was then replaced by the protein-free diet administered at the 90 calorie per kilo level.

CHART 3. The influence of pregnancy and lactation upon the ability of dog 4-3 to regenerate serum protein. The dotted lines represent plasmapheresis.
* During this preliminary period the dog subsisted on a natural stock diet followed by 1 week on an adequate "synthetic" casein ration.

Symbol	Blood volume	Hematocrit	Plasma volume	Period	Total bleeding		Serum protein removed
					Blood	Plasma	
				Dog 4-3			
	cc.	*per cent*	*cc.*		*cc.*	*cc.*	*gm*
A	892	40.5	531	B–C	425	248	11.4
C	920	40.7	546	C–D	0	0	0
D	763	40.0	458				
E	922	45.0	507				

During the periods when the animals were offered this ration, they refused at times to eat the daily aliquots completely. This necessitated feeding the dogs forcibly, a procedure which was tolerated very well, the ingested food always being retained. The casein diet was administered during the period of lactation at a level of 120 calories per kilo of optimal body weight and was always consumed with great avidity. Dog 4-3 weighed 13.6 kilos prior to term, 11.4 subsequent to

parturition, 11.2 when the protein-free ration was replaced by the casein diet and 11.5 when the last observation recorded on Chart 3 was made.   The corresponding weights listed for dog 4-4 were 15.8, 11.6, 10.6 and 10.2 kilos.

From the data given in the charts we note that these two dogs, as well as dog 5-6 described above, exhibited marked changes in the com-
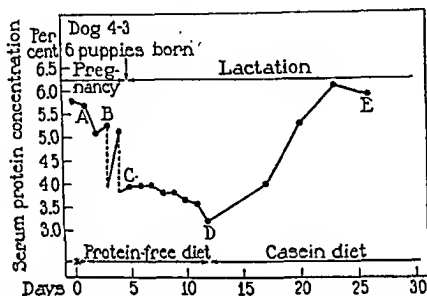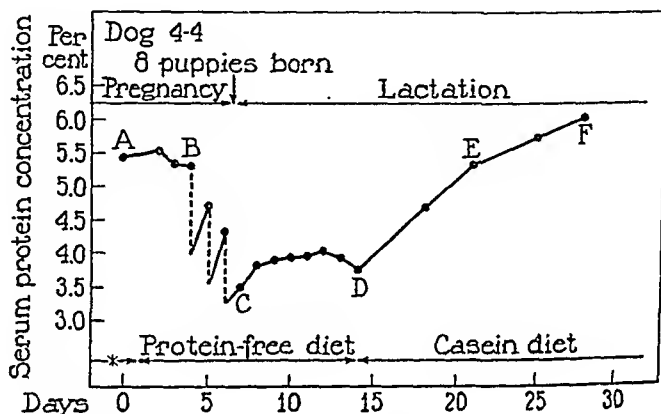

CHART 4. The influence of pregnancy and lactation upon the ability of dog 4-4 to regenerate serum protein.   The dotted lines represent plasmapheresis.

* During this preliminary period the dog subsisted on a natural stock diet followed by 1 week on an adequate "synthetic" casein ration.

Symbol	Blood volume	Hematocrit	Plasma volume	Period	Total bleeding		Serum protein removed
					Blood	Plasma	
Dog 4-4							
	cc.	*per cent*	*cc.*		*cc.*	*cc.*	*gm.*
A	904	30.8	626	B–C	683	455	20.9
C	1016	30.2	709	C–D	0	0	0
D	884	31.0	610				
E	873	30.5	607				
F	867	35 4	560				

position of the blood as a result of pregnancy.   Thus, dog 4-3 just before plasmapheresis was inaugurated, 2 days prior to parturition, had a serum protein concentration actually 26 per cent less than the normal value but despite this managed to have a normal plasma volume.   In the case of dog 4-4 there was a comparable decrease in

the serum protein concentration but actually a significant plasma hydration, so that the total amount of serum protein present in circulation was decreased by only about one-half as much (11 per cent) as in the case of dog 4-3. In both animals there were marked reductions in the hematocrit values and total cell volumes when comparisons are made with the normal findings recorded in Chart 2.

Of primary significance are the results which were obtained when the dogs subsisted on the protein-free diet and were subjected to the same standardized plasmapheresis procedures employed in the study described with the same animals in the normal state. In the case of dog 4-3 plasmapheresis was begun 2 days prior to parturition. Only two hemorrhages, resulting in the withdrawal of only 11.4 gm. of serum protein, were required to deplete this animal of its reserve serum protein and to lower the concentration of the blood protein to the basal level. These results should be compared with the normal findings (see Chart 2) which show that fully six bleedings, resulting in the removal of 38.4 gm. of serum protein, were essential for this purpose. During the subsequent week, period of early lactation, the serum protein concentration never did attain the basal bleeding level of 4.2 per cent but showed a gradual fall to the very low level of 3.2 per cent, when the animal's diet was changed to the adequate casein ration. In the normal state during a comparable period of time this animal had to be bled that amount of blood containing 7.6 gm. of serum protein in order to keep the concentration of the blood protein below the basal bleeding level. In like fashion with dog 4-4 only three hemorrhages were required to reduce the serum protein concentration to the basal level and, once attained, it was found not necessary to bleed the animal to maintain it there. The actual amounts of serum protein removed from this animal in the normal, in the pregnant and in the lactating states are given in Charts 2 and 4. These results are confirmatory of those obtained with dog 4-3. When both animals were fed the adequate casein ration and subjected to no bleedings the serum protein concentrations rose promptly during the period of lactation until normal values were approximated.

The data obtained from these experiments seem to gain in significance, since not only was each dog its own control but also because the

normal picture was obtained on one dog at the same time the effects of pregnancy and lactation were being evaluated on the other animal and *vice versa*. A period of approximately 7 months separated one study from the other. It is also pertinent to point out that experimental data are available to indicate that the results recorded for the initial depletion week and subsequent equilibrium periods are reproducible in the normal dog (15, 17).

One may feel that during the pregnancy study it was much easier to reduce the serum protein concentration to the basal level simply because the initial concentration values were already markedly less than normal. However, perusal of the protocols (26)[4] of a study conducted with dog 3 to determine the reproducibility of our procedures indicates that this could not be the only factor involved. The serum protein concentration of this animal was initially 6.21 per cent. One bleeding resulted in it being reduced to 4.82 per cent. However, in order to lower it to the basal level, it was essential to bleed the animal on the 5 successive days so that a total of 47.2 gm. of serum protein were withdrawn during this depletion period. Comparison of this value with that recorded initially with dog 3 (see Table I) indicates excellent agreement.

With both dogs the greatest change in the serum protein concentration occurred just at parturition. In the case of dog 4-3 it is obvious from the change in the plasma volume on that day that the decrease in the concentration of the blood protein cannot be attributed to hydremia. The blood volume estimation conducted on dog 4-4, however, did indicate a significant plasma hydration. We can find no reason to suspect that this particular determination is incorrect due to faulty technique; nevertheless, we feel that a significant decrease in the hematocrit value should also result if the hydremia were unquestionably so great. Since such did not occur and in view of the relationship between hematocrit and plasma volume in the subsequent determinations, it is concluded that the changes in the serum protein concentration in this dog were probably due primarily to our experimental procedures and not simply to a dilution of the plasma.

[4] Melnick (26), page 224.

The experiments described and discussed thus far indicate among other things that the pregnant dog possesses a limited store of reserve serum protein. In order to evaluate more completely the influence of pregnancy upon the regeneration of this blood protein additional tests were conducted on another animal. For this purpose plasmapheresis was initiated 2 weeks prior to term. It was also expected that this study would enable one to ascertain more fully whether or not the precipitous drop in the serum protein concentration observed previously is dependent specifically upon some peculiar effect of the parturition process. The results of the experiments are present in Chart 5.

The animal subsisted initially on the natural stock diet followed by 1 week on the synthetic casein ration. This was followed by use of the protein-free diet fed at the level of 90 calories per kilo of optimal body weight for a period of 17 days, when the litter was cast. Occasionally the diet had to be administered forcibly but here as in the case of dogs 4-3 and 4-4 the animal did not object to being fed in such a manner. Subsequent to parturition the casein ration was offered at a 120 calorie per kilo level and was always consumed with great eagerness. The weight of this dog was initially 10.8 kilos, rose to 12.3 at the time when the protein-free diet was initiated, continued to rise in spite of the inadequate ration to the peak value of 13.1 kilos but finally dropped to 12.4 just prior to parturition. Immediately after the litter was cast the dog weighed 10.7 kilos, which weight was maintained during the period of lactation. The optimal weight (24) of this dog was calculated to be 10.9 kilos.

The data presented in Chart 5 were obtained over a longer period of time than in the case of the other experiments. The finding of a plasma hydration and of marked decreases in the hematocrit value, in the total cell volume, in the serum protein concentration, and a negligible change in the total amount of serum protein were also recorded for this dog during pregnancy.

When subjected to plasmapheresis this animal showed precisely the same response as the pregnant dogs 4-3 and 4-4 when fed the same diet. Only three bleedings, resulting in the removal of 19.8 gm. of serum protein, were essential to deplete this dog of its reserve store of this protein and to reduce its concentration to the basal level. Subsequently for fully 12 days the serum protein concentration failed to reach the basal bleeding level of 4.2 per cent. It was not con-

sidered essential to obtain the normal control picture with this dog. On the basis of the average data presented in Table I, calculations indicate that this animal may be expected normally to lose about 33
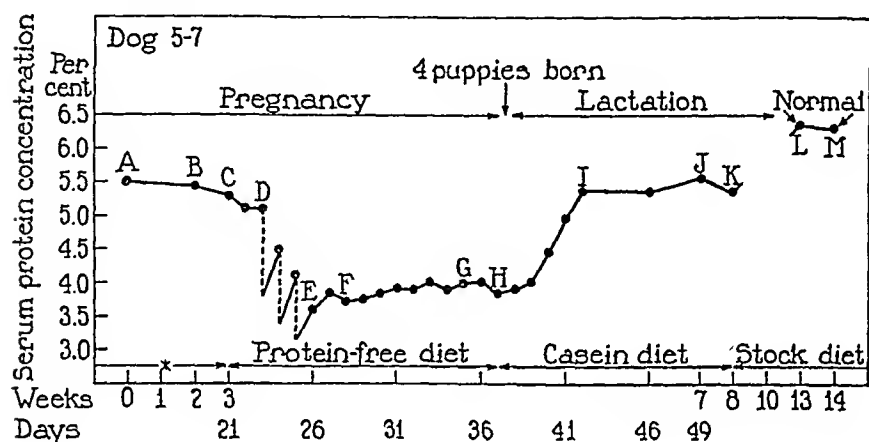
CHART 5. The influence of pregnancy and lactation upon the ability of dog 5-7 to regenerate serum protein.   The dotted lines represent plasmapheresis.

*During this preliminary period the dog subsisted on a natural stock diet followed by 1 week on an adequate "synthetic" casein ration.

Dog 5-7

Symbol	A	B	C	F	G	H	I	J	K	L	M
Blood volume, cc	1046	1014	940	918	935	939	848	926	915	925	951
Hematocrit, per cent	48.6	43.0	38.6	35.5	35.8	34.2	34.2	38.8	40.5	49.5	49.0
Plasma volume, cc	537	578	577	592	600	618	558	567	545	467	485

Period	D–E	E–H
Total bleeding, cc	703	0
Plasma, cc	452	0
Serum protein removed, gm	19.8	0

gm. of serum protein during the initial depletion week and approximately 7 gm. during the subsequent equilibrium period of 7 days. Of particular interest is the fact that this dog failed to show any greater increase in the hydration of the plasma at parturition.   Obvi-

ously then the ease, with which the serum protein concentration can
be reduced during pregnancy and lactation to the basal level and
maintained there, must be due solely to the parasitic effects on the
maternal organism of bearing the young and nursing them during
early life. In this connection it is interesting to note that dog 5-7
still evidenced during lactation a significant decrease of about 16
per cent in the serum protein concentration, even though it had sub-
sisted for almost 3 weeks on the adequate casein diet. The fact that
the total amount of serum protein during this period was normal
does not seem to be an adequate explanation; it is difficult to think
of an equilibrium between tissue protein and serum protein which
depends upon total quantities rather than upon concentrations.

DISCUSSION

From the evidence presented in our previous publications (17, 18,
15) a hypothesis was presented explaining the mechanism by which
serum protein formation takes place. This was illustrated by the
following diagram.
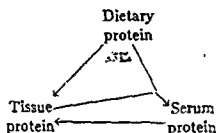
This hypothesis emphasizes the rôle of tissue protein catabolism as
an essential factor in the *normal* production of serum protein.

The results of our present investigations harmonize very well with
this concept. It has been reported that the amino acids of the blood
appear to be the precursors of the milk proteins (27) and that the
amino acids or polypeptides of the blood are utilized in the nutrition
of the fetus, since the placenta is apparently impermeable to the
plasma proteins (28). In our experiments the normal non-gravid
dog was shown repeatedly to be able to utilize the products of tissue
protein catabolism for the formation of serum protein. When fed
the protein-free diet and when subjected to quantitative plasma-
pheresis after the basal serum protein level is attained, the dog was

found still to be able to regenerate each week approximately 21 per cent of the total amount of protein normally present in the plasma. However, in the case of the pregnant and lactating animal subsisting on the same ration no appreciable regeneration of serum protein was observed to take place. Evidently under these experimental conditions, where there are no amino acids or polypeptides being absorbed from the gastrointestinal tract and where such products of tissue protein catabolism are utilized on the one hand for the nutrition of the fetus and on the other for the production of the milk proteins, the *Bausteine* of the serum protein complex are no longer available. It does not appear likely to us that the serum protein complex is the precursor of the milk proteins or the source of the amino acids essential for the nutrition of the fetus. It would seem to be very poor physiological economy for the organism to synthesize first the serum protein complex from the amino acids liberated as a result of tissue protein catabolism and then destroy it again to supply the amino acids for the functions of pregnancy and lactation involving additional protein synthesis.

In these studies of pregnancy in the dog we have observed no increase in the total blood volume, but in most cases a significant plasma hydration. This increase in the volume of the plasma occurred in spite of the markedly lowered serum protein concentrations. We believe one of the contributory factors for such a change in the plasma volume is the great reduction in the hematocrit and total cell volume. We have shown (29) with the *normal* dog that when there is a concomitant and significant decrease in the hematocrit resulting in a passive reduction of the blood volume, a lowered serum protein concentration is negligible in effecting a further decrease in the blood volume. In such conditions the plasma volumes were frequently greater than normal. Precisely the same variations in the composition of the blood appear to occur in the dog during normal pregnancy, so that the above explanation may very well be applied to explain in part such changes observed in the present study. Obviously some other factor must also be involved since shortly after parturition there is, especially in human beings, a marked decrease in the blood volume as a result of the removal of water from the plasma with a concomitant increase in the hematocrit due

simply to the hemoconcentration and not to any increase in the total cell volume.

SUMMARY

1. In view of the markedly short period of gestation in the dog and in view of the relatively large litters that are cast and nursed, this species when compared with the human being undergoes a much greater physiological strain during pregnancy and lactation. This is evidenced by marked decreases in the hematocrit values, in total cell volumes and in the serum protein concentrations, by an appreciable plasma hydration, and in some cases by significant reductions in the total circulating serum protein.

2. When pregnant dogs are fed a protein-free diet at a high level of caloric intake and are subjected to our standardized plasmapheresis technique, it is possible to deplete the animal of its reserve serum protein stores and reduce the serum protein concentration to the basal level (3.5 to 4.2 per cent) within the extremely short period of from 2 to 3 days. This indicates that the dog during pregnancy possesses a very limited amount of reserve serum protein.

3. Once the basal serum protein level is attained, the pregnant or lactating dog exhibits a marked impairment in its ability to regenerate serum protein. The synthesis of body proteins in the fetus during pregnancy and the milk proteins during lactation is considered to be actually an internal plasmapheresis, leading to a depletion of the serum protein by the preferential utilization of the materials from which this complex is made. These parasitic effects on the maternal organism are believed to be of primary importance, over and above any hydremia, in causing the lowered serum protein concentrations characteristic of pregnancy.

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STUDIES ON EXPERIMENTAL HYPERTENSION

VI. The Effect of Section of Anterior Spinal Nerve Roots on Experimental Hypertension Due to Renal Ischemia*

By HARRY GOLDBLATT, M.D., and WILLIAM B. WARTMAN, M.D.

(From the Institute of Pathology, Western Reserve University and University Hospitals, Cleveland)

It has been shown that, in the dog, neither section of the renal nerves (1, 2) nor excision of the thoracic portion of the splanchnic nerves and lower four thoracic sympathetic ganglia (3) can prevent or reduce the type of hypertension which can be produced in animals (dog and monkey) by constricting the main renal arteries by means of a special clamp (4, 5). Recently it has been reported (6) that even total thoracic and abdominal sympathectomy, including cardiac denervation, does not prevent or lower hypertension in dogs produced by the same method.

This report deals with the effect of section of anterior spinal nerve roots from the sixth dorsal to the second lumbar inclusive in preventing or reducing the persistent hypertension which can be produced in normal dogs by constricting the main renal arteries.

EXPERIMENTS

In four dogs, by a single operation on each, under ether anesthesia, the anterior nerve roots of the spinal cord from the sixth dorsal to the second lumbar inclusive were severed. This was effected through a dorsal midline incision and laminectomy. The mean blood pressure of these animals was determined during the entire period of the experiment by a direct method. This consisted of the insertion into the femoral artery of a 21 gauge needle connected to a mercury manometer by rubber tubing filled with 2 per cent sodium citrate. Stopcocks were so arranged as to permit the building up of pressure in the manometer from a reservoir

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of sodium citrate and, after the insertion of the needle into the artery, the instantaneous communication of the manometer with the interior of the vessel. If the femoral nerve is avoided, the procedure appears to be quite painless and the animal remains very quiet during the determination. Only mean blood pressure is determined by this method but it has the advantage of being entirely objective. The length of the control period before the operation for section of the anterior nerve roots varied from 4 to 6 weeks. Another control period of from 4 days to 2 weeks was allowed between section of the anterior nerve roots and the application of the clamp for constriction of the first renal artery. A short interval was also permitted between the operations for constriction of both main renal arteries.

RESULTS

In all four dogs the mean blood pressures either remained unchanged or fell slightly below their normal level for a varying period after section of the anterior nerve roots. In one dog, No. 3-03, it was still slightly lower, but in others it was at the original normal level at the time of constriction of the first renal artery. After permanent constriction of one renal artery, the mean blood pressure became slightly to moderately elevated in three of the four dogs. In one dog, No. 3-09, (Text-fig. 1) it remained unchanged during the 4 days which intervened between the clamping of the left and right main renal arteries. After permanent constriction of both main renal arteries, the blood pressure of the four dogs rose to a higher level and this elevation persisted or even increased. This is well shown in Text-figs. 1 and 2, which illustrate the mean blood pressure of dogs 3-09 and 3-10, respectively, throughout the entire experimental period. Dog 2-75 showed moderate elevation of mean blood pressure following the constriction of one main renal artery but it died before the clamping of the other artery was attempted. Dog 3-03 also developed elevated blood pressure, but after about 4 weeks it tended toward a lower level without actually returning to normal. This occurs occasionally in dogs with the renal arteries constricted and is probably due to the effect of the accessory circulation which may become very great and necessitates increased constriction of one or both renal arteries to re-elevate the pressure. In dog 3-03 the clamp on the left renal artery (the first one constricted) was tightened 3 months after it was applied. This resulted in re-elevation of the mean blood pressure which is now, 2 months later, usually from 65 to 75 mm. higher than it was before the application of the first clamp. All but dog 2-75 are still alive.
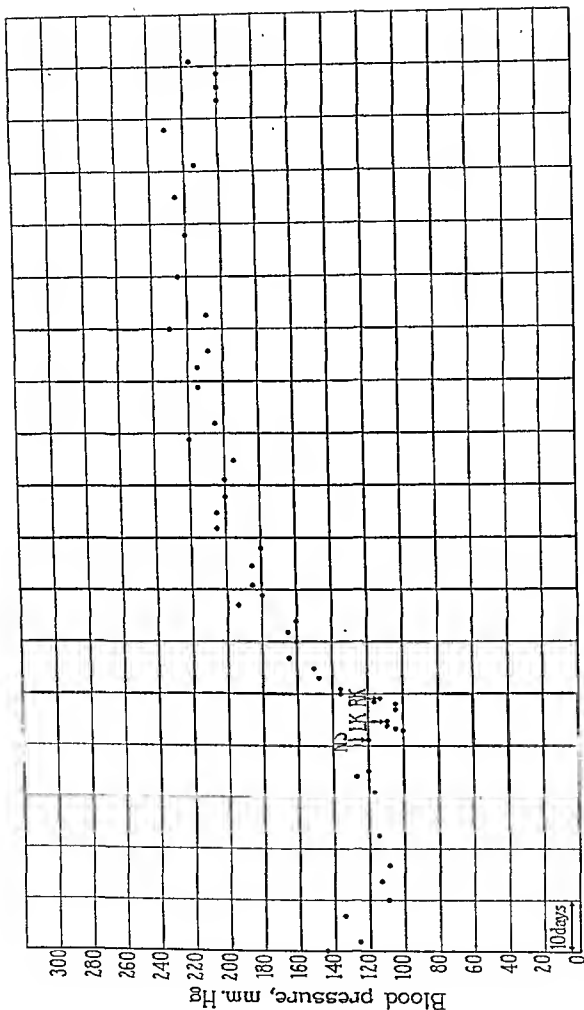
Text-Fig. 1. Dog 3-09. Female mongrel bulldog. Weight 10.2 kilos. NS, section of anterior spinal nerve roots from sixth dorsal to second lumbar inclusive. LK, constriction of left main renal artery. RK, constriction of right main renal artery. The mean blood pressure became elevated after constriction of both main renal arteries.

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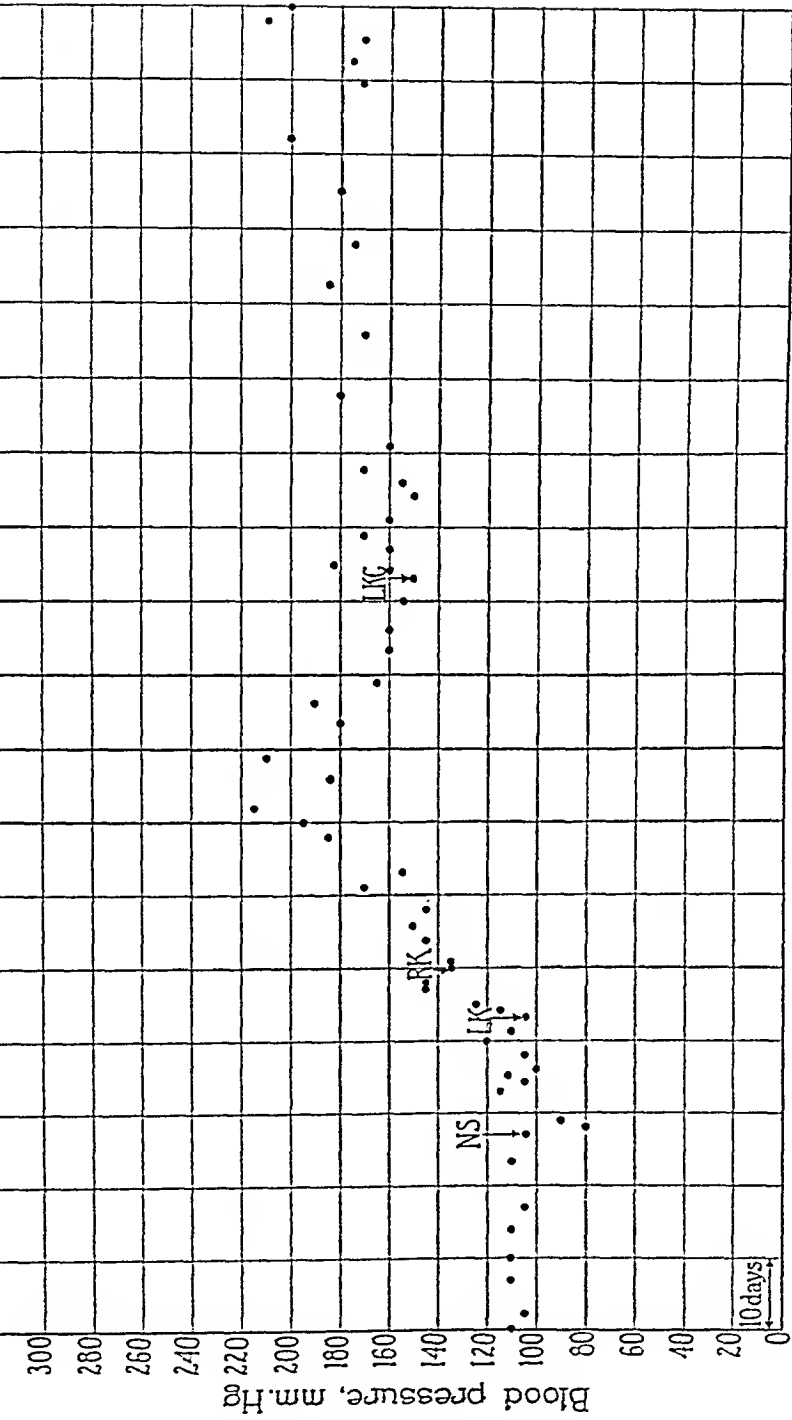
TEXT-FIG. 2. Dog 3-10. Female mongrel bulldog. Weight 10.6 kilos. NS. section of anterior spinal nerve roots from sixth dorsal to second lumbar inclusive. LK, moderate constriction left main renal artery. RK, moderate constriction right main renal artery. LKC, complete constriction left main renal artery. The mean blood pressure became elevated after the constriction of both main renal arteries.
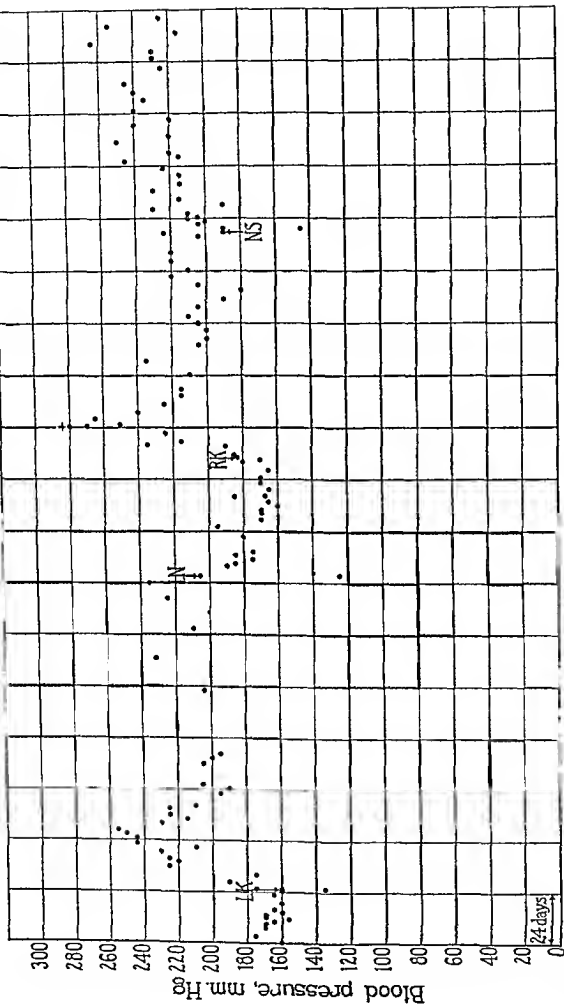
Text-Fig. 3. Dog 2-68. Female mongrel bulldog. Weight 15.2 kilos. LK, moderate constriction left main renal artery. LN, left nephrectomy. RK, moderate constriction right main renal artery. NS, section of anterior spinal nerve roots from sixth dorsal to second lumbar inclusive. +, more than 300 mm. Hg, mean blood pressure.
The mean blood pressure remained elevated after section of the anterior spinal nerve roots (NS).

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In two dogs, Nos. 2-68 and 2-35, hypertension was first produced and the anterior nerve roots were sectioned after the blood pressure had remained elevated for several months. In dog 2-68 (Text-fig. 3), the left main renal artery was first constricted. This was followed by the development of moderate hypertension which persisted, with a tendency downward, for about 5 months. As part of another study (7), the ischemic left kidney was then excised. This was followed promptly by a return of the mean blood pressure to the normal level and it remained at that level for 2 months. The main artery of the remaining right kidney was then constricted. The mean blood pressure rose to a very high level for a short time and then persisted at a lower but greatly elevated level. After 15 weeks of persistent hypertension, the anterior nerve roots from the sixth dorsal to the second lumbar inclusive were severed. The blood pressure has remained elevated after this procedure and has shown a gradual tendency upward (Text-fig. 3). The dog is still living. In the other dog, No. 2-35, systolic blood pressure was determined regularly throughout the entire experimental period by the van Leersum carotid loop method and occasionally by the direct method. The mean systolic pressure during a control period of 10 weeks was 158 mm. Hg. In this dog, bilateral splanchnic section within the thorax and excision of the lower four thoracic sympathetic ganglia failed to prevent the development of hypertension due to constriction of both main renal arteries (3). During the period between section of the splanchnic nerves and constriction of the first renal artery, the mean systolic pressure was 163 mm. Hg. After constriction of both main renal arteries, the mean systolic pressure was 244 mm. Hg. After this moderate degree of hypertension had persisted for more than 13 months, the anterior nerve roots from the tenth dorsal to the second lumbar inclusive were severed. There was a prompt fall of systolic and mean blood pressure after the operation and they remained lower for several weeks but did not fall to the normal level for this dog. Gradually the blood pressure has increased and now, 3 months after section of the anterior nerve roots, it is almost back to the high level at which it was before the section of the anterior nerve roots. During the last 2 weeks the systolic pressure has varied between 220 and 240 mm. Hg.

DISCUSSION

The failure of section of the anterior spinal nerve roots, from the sixth dorsal to the second lumbar inclusive, to prevent or reduce hypertension which can be produced by constriction of the main renal arteries (4, 5) is interpreted as due to the persistence of the effect of such constriction as long as the clamps remain applied. The same explanation applies to the failure of the other surgical procedures that have been carried out on the nervous system of dogs (1, 2, 3, 6) to affect this type of experimental hypertension, for none of these procedures has any effect on the clamp. These experiments make it very unlikely that the origin of this type of hypertension is due to a reflex from the ischemic kidney. They also minimize the part played by the nervous portion of the vasomotor apparatus of all parts of the body except the kidney in initiating the elevation of the blood pressure. Persistent or frequent stimulation of the vasoconstrictor nerves of the kidneys alone would probably produce hypertension by constricting the arterioles and reducing the blood supply to the functioning components of the kidney. The rest of the mechanism would be the same as that following constriction of the main renal arteries. This is being investigated at the present time. The probability is great that the mechanism whereby constriction of the main renal arteries results in hypertension is a humoral one, with a hypothetical effective substance of renal origin producing direct constriction of the muscle of the arterioles. This has been discussed at greater length in another publication (7). Since it is the narrowing of the arterioles and not of the main renal arteries that is associated with vascular hypertension in man, it is at least possible that section of vasoconstrictor nerves to the kidneys of hypertensive individuals would result in relaxation of the wall of renal arterioles in which the organic changes are not fixed. Improvement of the blood supply to the functioning components of the kidneys would occur. Lowering of blood pressure has been reported in about the same percentage of cases as a result of a number of different surgical procedures on the nervous system (8–17). Common to all is section of the vasoconstrictor nerves to the kidneys. This indicates that in all cases the beneficial effect may be due to

the relaxation of renal arterioles and improvement of circulation to the functioning components of the kidneys and not, as has been suggested, to the relaxation of arterioles in a large part of the vascular bed in the abdomen. The failure of these same procedures to affect experimental hypertension due to constriction of main renal arteries does not in any way controvert the results obtained in human hypertension. It merely emphasizes the importance of reduced blood supply to the kidneys in the pathogenesis of this type of experimental hypertension and perhaps in human hypertension that is associated with renal arteriolar sclerosis.

SUMMARY

Section of the anterior spinal nerve roots from the sixth thoracic to the second lumbar inclusive did not prevent or significantly and permanently reduce hypertension produced by constricting the main renal arteries of dogs. The significance of these results for the pathogenesis of human and experimental hypertension is discussed.

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ON THE MODE OF ACTION OF SULFANILAMIDE IN EXPERIMENTAL STREPTOCOCCUS EMPYEMA*

By FREDERICK P. GAY, M.D., AND ADA R. CLARK, PH.D.

(From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York)

PLATE 14

Little doubt remains as to the chemotherapeutic activity of the substance now conveniently designated sulfanilamide (para-amino-benzene-sulfonamide) and its derivatives, in experimental and naturally occurring infections due to the streptococcus and perhaps other living disease agents. Some form of this drug would seem destined to assume an as yet not fully defined place in the treatment of infections. An increasing appreciation of the therapeutic possibilities of this drug has followed rapidly on the striking experimental results of Domagk in 1935 (1). These results, however, were arrived at empirically, as has been the case with the discovery of most of the chemotherapeutic agents, and further analysis along practical lines has outstripped any definite information as to the precise method by which this substance acts therapeutically in the animal body.

The very fact that this particular drug, although highly effective in the body, has no bactericidal effect on the streptococcus when added to nutrient media in the test tube, a fact that we have ourselves repeatedly verified, at once points to a necessary adjuvant or determinative action on the part of host fluids or host cells. Despite extended investigation, knowledge of the mechanism of host participation in chemotherapeutic action in general, has lagged far behind practical therapeutic results (cf. 2). In the particular case that we are considering the possibility of cell intervention in sulfanilamide action has been considered from the very beginning. Domagk (1) noted an increase of mononuclear cells in the peritoneal exudate of

treated mice, and thought that the reticulo-endothelial system might participate more fundamentally in the drug action. Colebrook and Kenny (3) mentioned a similar possibility without experimental confirmation. Levaditi and Vaisman (4) also noted a macrophage increase but found that blockade by colloidal copper combined with splenectomy in mice did not decrease the chemotherapeutic action. A similar negative effect of splenectomy alone has been described by Gross, Cooper and Peebles (5). With less well defined emphasis, Rosenthal (6) and Long and Bliss (7) have mentioned increased phagocytosis as probably adjuvant to the action of sulfanilamide but this has been specifically denied by Mellon, Gross and Cooper (8).

Our long experience (9) in the study of experimental streptococcus infections in animals has led us increasingly to the conviction of the importance of the slowly mobilizable cells of the macrophage series in natural resistance and in acquired immunity to this microorganism. It was natural then that in analysis of a presumable cell intervention combined with a chemotherapeutic agent, particularly one that is specifically active against the streptococcus, we should turn to an experimental syndrome, streptococcus empyema in the rabbit, which has served as a basis of our study for many years.

EXPERIMENTAL

We have repeatedly described the method we have employed in provoking an extending and rapidly fatal empyema by injecting minimal amounts of a culture of hemolytic streptococcus "H" originally derived from man and still retaining its human characteristics (10) in spite of passage for nearly twenty years through the pleural cavities of some 200 rabbits. This culture in a dosage of not over 10 chains in an 18 hour broth culture (dilution 10^{-7}) seeded directly from the conserved pleural fluid of a fatally infected rabbit usually kills in 4 to 6 days, on direct intrapleural inoculation. The virulence has remained relatively fixed for several years.

Sulfanilamide[1] when given rabbits in relatively large amounts, be-

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[1] We have employed for the most part the preparation known as prontylin (para-aminophenylsulfonamide) which the Winthrop Chemical Company has kindly prepared for us in crystalline form without the excipient employed in the tablets designed for oral administration.

ginning a few hours before intrapleural infection with 1000–2000 M.L.D., and continued for at least seven doses during the first 2 days, aborts the otherwise fatal empyema. It requires three daily subcutaneous doses of 20 cc. each of a 2 per cent solution of the sulfanilamide crystals dissolved in boiling water and cooled to body temperature, that is to say, 1.2 gm. of the drug daily, for at least 2 days or a total of almost 3 gm., to effect complete protection against the streptococcus. Smaller total amounts of the drug will at times protect, that is to say, will lead to the complete sterilization of the pleural cavities and blood stream, but assured protection requires the larger dosage indicated. Even larger total amounts of the drug may be given without fatal result although they produce well defined symptoms such as respiratory difficulty, reduction of the red blood cells and loss of weight. We have, for example, administered a total of 10.8 gm. of sulfanilamide to a 2400 gm. rabbit within 10 days without fatality.

Obviously we are dealing here only with a preventive action induced by sulfanilamide and not with a true curative effect. We have found, to be sure, that a rabbit in which treatment was begun 24 hours after infection survived for 11 days as contrasted with the uniform death of controls in 4 to 6 days. Another rabbit, with treatment beginning 48 hours after infection, died in 5 days, like the control. Our interest at this point lies solely in the study of the mechanism involved under conditions in which the drug is effective, irrespective of any practical therapeutic bearing it may have.

Action of Sulfanilamide as Tested with the Serum of Treated Animals in Vitro

It is generally agreed that sulfanilamide and its derivatives have little if any direct effect on streptococci when added to culture media. On the other hand it has been found that the blood of man and animals that have been treated with the drug may have a distinct inhibitory effect on the microorganism as contrasted with normal blood. As illustrated in our own experiments this bacteriostatic effect is transitory and never results in complete destruction of even a few chains of streptococci.

Experiment 1.—0.1 cc. of a 1–1,000,000 dilution of an 18 hour streptococcus "H" culture (15 chains) was added to 1 cc. of the fresh blood serum of a normal rabbit. The same amount of culture was added to the serum of a rabbit that had been given four 20 cc. doses of 2 per cent sulfanilamide, from 26 to 3 hours before obtaining the blood. 0.1 cc. of these culture mixtures was plated out at intervals on blood agar in successive dilutions and the resultant colonies counted.

TABLE I

Action of Fresh Serum from a Sulfanilamide Treated and a Normal Rabbit on Streptococcus "H"

	Number of chains per cc.			
	5 hrs.	15 hrs.	44 hrs.	92 hrs.
Treated rabbit's serum 1 cc. + 0.1 cc. (10⁻⁶) broth culture streptococcus "H" (±15 chains)	4000	12,000,000	11,000,000	60,000,000
Normal rabbit's serum 1 cc. + 0.1 cc. (10⁻⁶) broth culture streptococcus "H" (±15 chains)	11,500	500,000,000	370,000,000	90,000,000

It is evident from Table I that although the streptococci are initially (44 hours) inhibited in growth over the control, the number of colonies at the end of 4 days is practically the same.

This contrast in action of drug treated rabbit's serum can be definitely increased if whole defibrinated blood or aseptically produced pleural fluid is employed and if the tubes are continually agitated in a shaking machine. In no instance, however, does the culture become sterile or is the resulting number of colonies at the end of several days remarkably different from controls with normal fluids.

An experiment with serum only has been used for illustration as we wished to contrast this bacteriostatic effect *in vitro* of the fluid of the treated animal free from its cells, with what takes place in the body of a similar animal.

In all instances successful abortion of the rabbit empyema syndrome depends on the repeated injection of the drug and one might well question whether successive doses of sulfanilamide treated serum to a given culture dilution might not result in complete sterilization.

Experiment 2.—0.1 cc. of a 1–1,000,000 dilution of an 18 hour streptococcus "H" broth culture (15 chains) was added to 1 cc. of fresh blood serum of a normal

rabbit. The same amount of culture was added to the serum of a rabbit that had been given four 20 cc. doses of 2 per cent sulfanilamide, from 26 to 3 hours previous to obtaining the blood. At six intervals over a period of 6 days, 0.5 cc. of each mixture was plated out on blood agar in successive dilutions and the colonies counted. Following removal of the serum for plating, 0.5 cc. of fresh corresponding serum, either control or drug-treated, was added to the remaining culture mixtures.

TABLE II

Effect of Successive Additions of Fresh Serum from a Sulfanilamide Treated and a Normal Rabbit on Streptococcus "H"

	Number of chains per cc.					
	5 hrs.	15 hrs.	44 hrs.	92 hrs.	120 hrs.	144 hrs.
Treated rabbit's serum 1 cc. + 0.1 cc. (10^{-4}) broth culture streptococcus "H" (±15 chains)	2600	3,000,000	1,000,000	95,000	1,100,000	65,000,000
Normal rabbit's serum 1 cc. + 0.1 cc. (10^{-4}) broth culture streptococcus "H" (±15 chains)	3700	26,000,000	480,000,000	90,000,000	400,000,000	500,000,000

This experiment shows clearly that although successive additions of drug treated serum result each time in fresh inhibition of the streptococcus growth, and in spite of the fact that the total number of organisms is divided in half before each addition of serum, the culture fails to become sterile. It is clear then that sulfanilamide cannot produce its maximal therapeutic effect simply as a chemical dissolved or transformed in the fluids of the body.

The streptococcus that has been checked in its development in sulfanilamide serum shows distinct degenerative changes that are illustrated in Fig. 2. The chains elongate markedly and the individual cocci are swollen, and metachromatic in the sulfanilamide serum culture, as contrasted with their growth in normal serum (Fig. 1). These distorted cells, however, are still to a degree capsulated. Al-

though the sulfanilamide treated cocci on initial plating may show more "matt" colonies than the "mucoids" that are characteristic of the strain grown in normal serum, on subculture the matt colonies revert at once to mucoid.[2] Much more important is the fact that the culture treated repeatedly with the serum of sulfanilamide treated rabbits has lost none of its virulence.

Thus in two different experiments cultures of streptococcus were prepared by growth in successive additions on the one hand of fresh normal rabbit serum, and on the other, of serum of a rabbit given several doses of sulfanilamide in the manner just described. After determination of the number of viable organisms in each culture mixture, dilutions were made in such a way as to insure approximately the same number of chains of the two different organisms.

A series of rabbits was inoculated with dilutions at 10^{-7}, 10^{-6} and 10^{-5} of the two cultures; that is, 10–13 chains, 100–130 chains and 1000–1300 chains. The two rabbits given the smaller amounts of the two cultures survived, whereas those with the more concentrated dilutions died of typical pleurisy. There is evidently then no essential loss in virulence in a sulfanilamide-serum treated streptococcus.

Action of Sulfanilamide in Rabbit Empyema

Preventive and continued treatment of rabbits with sulfanilamide in sufficient doses prevents the evolution of experimental streptococcus pleurisy as already stated. It remains to follow in more detail the changes that accompany this disappearance of the streptococci in the body of the infected animal.

For the purpose several series of rabbits, both normal and treated, were infected intrapleurally with comparable multiple doses (1000–10,000 times the lethal dose) of streptococcus "H," and killed at intervals of 12, 24, 36, 48, 60 and 72 hours, that is to say, up to the period just before death naturally occurred in the controls. Treated animals were killed at later intervals to compare the findings with those of the controls as the latter died.

The examinations included repeated leucocyte counts of the blood during life in selected cases. At death or on sacrifice of the animal cell counts were made on the exudate or washings from the infected pleural cavity with estimation of

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[2] We owe these observations to Mrs. Dorothy W. Miles.

the relative proportions, total number, and the condition of polymorphonuclear and mononuclear cells. Cultures were not only taken from both pleural cavities and the blood stream to indicate the extension of the process, but in the case of the infected pleural cavity the total number of living chains of cocci was estimated by plating. Histological studies were made in particular of both the visceral and the parietal pleura and in several instances of other organs, particularly of the liver, spleen and bone marrow. We present herewith a mere summary of the results obtained in these examinations.

Changes in the Blood.—In control non-treated animals the cultures from the blood have been found positive for streptococcus in every instance from 12 hours onward. There is a drop in the total leucocyte count from 20 hours onward of from one-third to one-half the original number, and from 24 hours onward distinct degenerative changes were noted in the polymorphonuclear cells. From 48 hours onward the mononuclear cells increase to from two to five times the original number.

In sulfanilamide treated animals the blood cultures are uniformly sterile. There is an increase in the total leucocyte count during the early period of infection which reaches from one and one-half to twice the original number of cells up to a period of, roughly, 40 hours. No degenerative changes in the polymorphonuclears were noted. From 48 hours onward, as in the controls, the mononuclear cells increase from two to three times the original number. This relative and actual increase in mononuclears persists long after the infected pleural cavity has become sterile and the total leucocyte count has returned to normal.

The Infected Pleural Cavity.—In the control untreated animal streptococci injected in the pleural cavity increase rapidly. In an animal killed in 12 hours, 6500 times as many organisms were found as had been injected; another at 24 hours gave the same number of multiples; in 48 hours estimates in two animals gave 8000 and 20,000 times the original number. Cultures from the left (uninoculated) cavity were positive in all instances from 12 hours onward. The amount of fluid in the cavity is found markedly increased from 24 hours onward to the time of death, the range being from 2 or 4 cc. in 24 hours to 15 or 20 cc. at the end of 6 days. Throughout the series the cells that compose this exúdate are predominantly polymorphonuclear, the

mononuclear clasmatocytes, so long as differentiation can be made, giving relative percentages of from 3 per cent to 16 per cent (average of 8). From 48 hours onward the polymorphonuclears are degenerated so as not to be recognizable except by contrast with the mononuclear cells which may still stain fairly well for at least 2 days.

In the sulfanilamide treated animals the pleural cavity reacts quite differently. In contrast to the immediate increase of cocci in the control to 6500 times in 12 hours, in the treated animal a single animal showed an increase of only 10 times. In the 24 hour animals all three that were examined were positive in the right cavity but in two of these that were plated out the increase was ×3 in one and a decrease in the other to such an extent that 0.1 cc. of the 1 cc. of broth used to wash the clean cavity gave no colonies on a plate, although a slightly larger amount in broth finally became positive. A single 36 hour animal gave exactly the same result as this last animal. Of three animals killed at 48 hours two were completely sterile and one gave a reduction to 150 colonies for the entire cavity from the 37,000 originally introduced. All subsequent cultures from the right cavity in fully treated animals that were observed from 72 hours onward remained completely sterile. With the exception of one animal killed at 12 hours and another at 48 hours, the left (uninoculated) cavities never yielded positive streptococcus cultures.

The amount of exudate in the inoculated pleural cavities of the treated animals was in sharp contrast to that observed in the control animals. In only one instance (48 hours) was any measurable amount of fluid present; estimates in the others, both of the streptococcus and of the exudate, were made by introduction, agitation and removal of a small amount of sterile bouillon. The relative cell counts showed a comparatively small number of polymorphonuclear cells throughout this series since the clasmatocytes ranged from 6 to 73 per cent (average 33 per cent) during the sterilizing period of 48 hours. This resembles the findings noted by Gay and Morrison (11) who found that rabbits protected by preparation with broth showed essentially normal pleuras after infection, although in this case the sterilization (11)[3] was accomplished within 24 hours. A further

[3] Gay and Morrison (11), table 6.

contrast between the exudates in treated and untreated animals, apart from the volume and the relative proportions of cells, lies in the superior condition of both types of cells in the treated series. This is evident even in the exudates of animals insufficiently treated with sulfanilamide that die after the control death period of streptococcus invasion. In the control animals the more labile polymorphonuclears begin to show distinctive degenerative changes as early as 24 hours although the lower percentage of clasmatocytes remain relatively intact for 24 hours longer. Throughout the treated series both types of cells remain relatively normal in appearance. In the treated series phagocytosis by mononuclears is evident and red-staining (dead) chains are notable (Wright stain). This is apparent in spite of the fact that the restricted number of cocci present in the treated animals makes the organisms difficult to find.

Histological Basis of the Resistance Induced by Sulfanilamide

Our previous studies (12) on enhanced local resistance of the pleura to virulent streptococcus, particularly as produced by various inert substances such as broth, aleuronat and gum arabic, have clearly demonstrated that it is due to accumulations of mononuclear cells in adjacent tissues. These results have been amply confirmed by numerous observers. It is natural then that we should have sought for histological changes, whether local or general, in rabbits protected from streptococcus empyema by means of sulfanilamide. Our previous studies had led us to the conviction that the mononuclear cells accumulated in the subserous layers of the pleura were largely local in origin, that is to say, developed from the clasmatocytes (Ranvier), or tissue macrophages, of the connective tissue. We have found no evidence of passage of these cells through the general circulation from more remote areas of the reticulo-endothelial system. We confess to no profound study of such tissues as the spleen, liver and bone marrow in search of a possible remote origin of such cells in our previous work. In connection with this work on sulfanilamide we have, however, examined not only the circulating leucocytes in infected and normal animals, with and without drug treatment, but also the organs that have been mentioned. We have found no histological evidence from this study that sulfanilamide acts through

a general stimulation of the reticulo-endothelial system, a conclusion in agreement with the blockade and splenectomy studies of others that have been mentioned in the introduction.

There remains, however, the possibility that sulfanilamide itself stimulates the local accumulation of mononuclear cells in the pleural wall whenever an irritant is injected into the pleural cavity. To test this possibility we have undertaken two experiments.

Experiment 3.—Twelve adult rabbits were given each 3 cc. of 5 per cent aleuronat plus 3 per cent starch in the right pleural cavity. Six of these rabbits were given subcutaneous injections of sulfanilamide (20 cc., 2 per cent) three times daily beginning 4 hours previous to the aleuronat injections. The other six received none. The drug injections were continued until 3 hours before the animals were sacrificed. Three animals of each series were killed within 24 hours after the aleuronat injections; two each at 48 hours, and one each at 72 hours. The exudates and sections from lung and parietal pleura were studied from the viewpoint of total numbers and relative properties of cells.

The histological picture on comparing these two series was indistinguishable, except as regards individual variations. In 24 hours in both sulfanilamide and control series the exudate and the subserous accumulations of cells are predominantly polymorphonuclear; in 48 hours the cells are mixed, and in the 72 hour animals mononuclear cells predominate. If anything, the mononuclear cells in the nontreated aleuronat series exceeded on an average those in the sulfanilamide aleuronat series. The conclusion is that this drug does not accelerate or increase mononuclear cells in the pleural wall when a sterile irritant is used.

The further possibility exists, however, that the peculiar relationship between mononuclears, sulfanilamide and streptococcus might give different results if streptococci were the irritant employed. To test this possibility we undertook a further experiment.

Experiment 4.—In this double experiment, a small series, three each, of sulfanilamide treated and untreated rabbits were given 500 million formalin killed and washed streptococci in the pleural cavity and representatives of each series sacrificed at 13, 24 and 48 hours. In a second group of treated and untreated animals, two injections of killed streptococci were given at intervals and the animals in pairs were sacrificed 6 hours after the last streptococcus injection.

Exudates and sections from the parietal pleura from the two series gave individual differences that seemed notable but that were not consistent as contrasting between the two series. We are unconvinced that in this particular experiment sulfanilamide treatment showed distinctive stimulating effect for mononuclear cells even in the presence of streptococcus protein.

The failure to demonstrate any distinctive mobilizing power for mononuclear cells on the part of sulfanilamide when the pleural cavity is irritated either by an indifferent substance or by streptococcus protein, renders the consistent histological findings in infected animals cured by sulfanilamide more striking.

We have studied with particular completeness and in sufficient numbers the pleural tissues (visceral and parietal) throughout the critical period (12 to 72 hours) during which cure is established in the sulfanilamide treated animals, and by the end of which time deaths began to occur in the controls.

At the 12 hour stage the only change noted in the tissues in representatives of both series was congestion with slight hemorrhage of the vessels of the serosa but without distinctive cell accumulations.

At 24 hours a difference in the two series begins to appear. Although polymorphonuclear cells predominate in both they are present in larger numbers in the sulfanilamide animals and are found deeper down among the muscle bundles of the parietal wall. A considerable number of mononuclear cells was also noted in one of the treated animals. The sharp histological differentiation begins, however, at 36 hours and continues onward with increasing emphasis.

In the control animals the polymorphonuclears predominate throughout the life of the animal and become increasingly degenerated until death. Necrosis in the muscle bundles begins to appear. Although mononuclear cells appear in small numbers and are at first relatively intact they too become degenerate in appearance by 72 hours (Fig. 3).

Among the treated animals a few mononuclears are evident as early as 24 hours and from 36 hours onward they are the increasingly predominant cell. They are found massed deep down among the muscle bundles and particularly along the septa as well as in the sub-

serous layer of connective tissue which has thickened to accommodate them. The subserous layer of the visceral pleura (Fig. 4), which is very thin normally, thickens notably and nodules of mononuclear cells accumulate around the adjacent alveoli. These cells (septal) are at times in active mitosis. These mononuclear cell accumulations are not so striking at the exact period (48 hours) when complete sterilization of the cavity has just occurred and they are still interspersed with polymorphonuclear cells. They become more notable from 4 days onward (Figs. 5, 6 and 7) and most marked of all in those cases where death was merely delayed until the 9th or 11th day through inadequate treatment.

DISCUSSION AND CONCLUSIONS

Sulfanilamide prevents the evolution of an invariably fatal streptococcus empyema in rabbits when it is given repeatedly and in sufficient doses subcutaneously. Complete sterilization of the inoculated cavity occurs on approximately the 2nd day. The serum, defibrinated blood and artificial pleural exudate of similarly treated animals inhibits the growth of the same streptococcus in the test tube but even repeated doses of such treated blood serum fail to sterilize the culture. The coccal chains grown in such drugged serum are elongated and present pleomorphic and metachromatic organisms and may give rise to colonies that are at first less predominantly mucoid in appearance. Such organisms have, however, lost little if any of their virulence.

Cooperation on the part of locally derived clasmatocytes is apparently required in complete sterilization of the animal body. This conclusion is reached not only by a process of exclusion from comparison with the test tube results, but through the direct histological demonstration of a precocious and increasing mobilization of clasmatocytes in the parietal and visceral pleura of treated animals.

In other words, sulfanilamide apparently produces a bacteriostasis sufficiently marked to protect the accumulated leucocytes and to allow the natural defense macrophages to accumulate. There is direct evidence that the drug does not in itself stimulate the mobilization of the macrophages. There is no evidence that the cell reaction

which finally accounts for disposal of the organisms is other than local.

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EXPLANATION OF PLATE 14

FIG. 1. Streptococcus "H," grown for 92 hours in three successive additions of fresh normal rabbit serum.   × 1700.

FIG. 2. Streptococcus "H," grown for 92 hours in three successive additions of fresh serum from a sulfanilamide treated rabbit.   × 1700.

FIG. 3. Visceral pleura and lung of untreated control rabbit 16-75.   Killed 48 hours after intrapleural infection with streptococcus.   Zenker (without acetic acid) fixation.   Eosin and methylene blue.   × 96.

Extensive polymorphonuclear exudate on surface of the lung.   Serosa merely suggested by wavy line.   Infiltration of subserous layer and adjacent alveoli by polymorphonuclears.

FIG. 4. Visceral pleura and lung of sulfanilamide treated rabbit 7-06.   Killed 48 hours after infection with streptococcus.   Fixation, staining and magnification (× 96) as in Fig. 3.   No exudate.   Serosa intact.   A slight but almost entirely mononuclear infiltration of cells in subserous layer.

FIG. 5. Parietal pleura of control rabbit 16-67.   Death 90 hours after infection with streptococcus.   × 144.

A thick necrotic exudate on surface with no intact cells left.   Few cells in subserosa, mostly polymorphonuclears, and for the most part with pyknotic nuclei.   Adjacent muscle bundles necrotic.

FIG. 6. Parietal pleura of rabbit 16-64 treated with sulfanilamide and killed 90 hours after infection.   Cultures all sterile.   No exudate.   Serosa somewhat thickened.   Moderate infiltration of mononuclear cells in widened subserous layer.   Nests of mononuclear cells between normal muscle bundles.   × 144.

FIG. 7. Visceral pleura of same rabbit (No. 16-64) as in Fig. 6.   × 412.

No exudate.   Serosa normal.   Dense masses of mononuclear cells in subserous layer apparently arising from alveolar walls.   Terminal bronchi (not shown in illustration) are in places filled with mononuclear cells.

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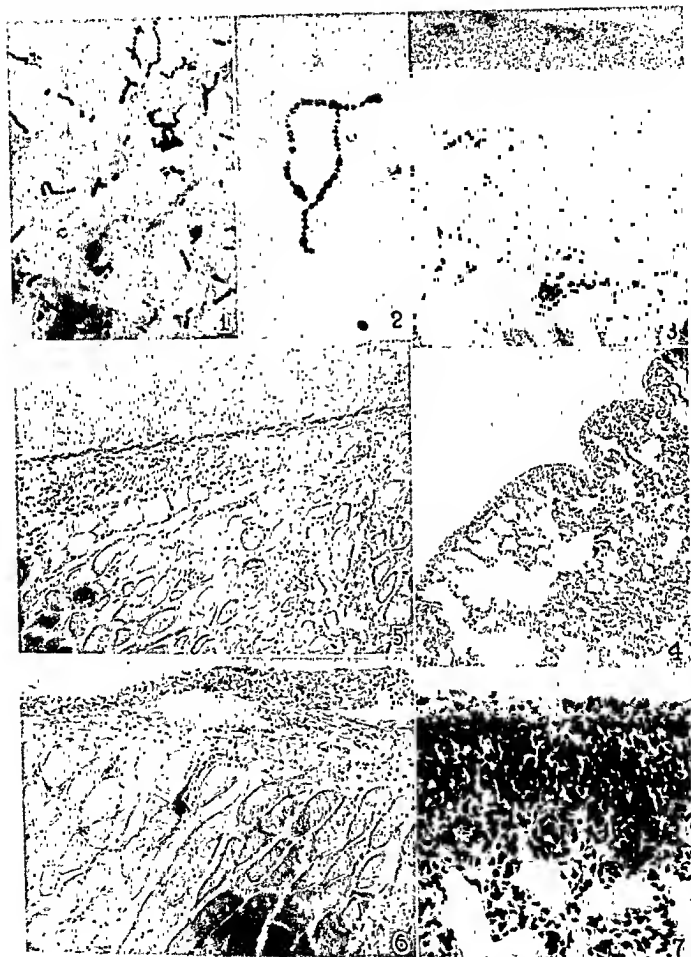
STUDIES ON THE RÔLE OF THE SPLEEN IN EXPERIMENTAL POLIOMYELITIS*

By EDWIN H. LENNETTE, M.D.

(From the Department of Bacteriology and Parasitology, The University of Chicago, Chicago)

The cellular aspects of immunity to poliomyelitis, almost completely overshadowed by the intensive investigations conducted on its humoral features, have received far less attention than they merit. Leiner and von Wiesner (1) were unable to detect poliomyelitis virus in the spleen, and Levaditi (2) states that the virus is not found in the kidney, bone marrow, liver or spleen. Flexner and Amoss (3), however, found that the virus survived in the spleen for at least 17 days after intravenous inoculation, but that its "virulence" had been diminished. Brebner (4) stressed the fact that many intracerebral lethal doses of virus can be injected directly into the spleens of normal monkeys with little danger of infection resulting, but that similar injections into immune monkeys are followed by a severe, possibly anaphylactic, reaction and death (5).

The experimental work reported here is concerned with the effect of splenectomy on resistance to poliomyelitis and with the rôle of the spleen in fixing and disposing of the virus. *Rhesus* monkeys (*Macaca mulatta*) were used in all the experiments.

Effect of Splenectomy on Resistance to Infection

The functional rôle of the spleen in resistance to infection has been the subject of numerous investigations; as a result of these studies it is generally accepted that this organ is intimately associated with immune states, natural or acquired. Most investigations, however, have been concerned with bacteria or other large

forms, such as the blood protozoa, the filterable viruses receiving little consideration.

The importance of the spleen in an infection due to a highly neurotropic virus such as that of poliomyelitis, which apparently follows a direct course from the nasal mucosa to the central nervous system *via* the olfactory nerves (6), depends a great deal on whether the virus remains confined to its neural pathways or whether it circulates in the blood at any period during the pathogenesis of the disease. Since attempts to demonstrate poliomyelitis virus in the blood stream following its intracerebral or intranasal administration have seldom been successful (7), and since introduction of the virus by either of these routes is practically always followed by the clinical disease, we resorted to intravascular inoculation, which is effective only when large amounts of virus are injected.

Methods

Test monkeys were splenectomized under deep ether or nembutal anesthesia 2 to 3 weeks prior to inoculation, so that the inflammatory reaction about the incision would have an opportunity to subside and healing occur, thus preventing possible infection of local nerve tissue. Splenectomized convalescent or immunized monkeys and non-splenectomized normal monkeys were used as controls.

The MV strain of virus was used in all the experiments here reported, the inoculum being prepared by grinding pooled, glycerolated poliomyelitic spinal cords with sufficient physiological salt solution to give a 10 per cent suspension, which was then centrifuged at 2,000 R.P.M. for 30 minutes. The markedly turbid supernatant was drawn off, filtered through paper and injected intravenously; inoculation was done very slowly through a fine needle, since such preparations were found to be quite toxic and occasionally caused an anaphylaxis-like reaction with death.

When large amounts of virus (2 or 3 daily injections, see Table I) were given intravenously to non-splenectomized control monkeys, half the animals (9 of 18) became infected, while the same proportion of splenectomized monkeys (4 of 9) succumbed when given similar treatment. None of 14 immunized or convalescent animals without spleens reacted to these large doses of virus. On the other hand, when smaller doses of virus (single injections, Table I) were administered to 6 normal monkeys as controls, none showed any reaction, whereas 2 of 5 monkeys previously splenec-

tomized became paralyzed.[1] This was the only evidence that splenectomy played any part in lowering the resistance of the experimental animal to intravenously injected virus.

These results suggest that when large doses of virus are used infection possibly results from an overwhelming of the defensive mechanisms of the nasal mucosa (8) or from penetration of the virus through some other barrier (9), regardless of whether or not the spleen is present, and is a chance occurrence. Conversely, small amounts of virus may fail to infect the intact animal because they are quickly fixed by the spleen and so fail to reach the central nervous system,

TABLE I

Effect of Splenectomy on Infection by the Intravenous Route

Description	Virus dosage (10 cc.)		Number of monkeys inoculated	Poliomyelitis	
	Injections	Virus		Number positive	Number negative
		per cent			
Splenectomized immune and convalescent monkeys	2 or 3	5 or 10	14	0	14
Splenectomized normal monkeys	2 or 3	5 or 10	9	4	5
	1	10	5	2	3
Non-splenectomized normal monkeys	2 or 3	5 or 10	18	9	9
	1	10	6	0	6

whereas in the absence of the spleen the virus may possibly circulate freely for a sufficiently long interval to reach the nervous system and initiate infection.

We next attempted to determine the effect of splenectomy subsequent to inoculation. The intravenous and intrasplenic routes of inoculation were used.

Six monkeys were given one intravenous injection of 10 cc. 10 per cent virus suspension and pairs of them splenectomized at 1, 3 and 5 day intervals after the injection. Observations and temperatures were recorded daily for 4 weeks, but no reaction to the virus was noted at any time (Table II). Splenectomy, therefore, did not

[1] In another connection, we have recently injected a series of 23 intact normal monkeys with similar single doses of virus, and in no case did paralysis ensue.

render these animals susceptible to doses of virus subinfective for non-splenectomized monkeys.

Using the intrasplenic route, essentially the same results were obtained (Table III). The spleens were exteriorized to minimize leakage of the inoculum into the peritoneal cavity or subcutaneous tissues and to avoid entering the belly twice. Exteriorizing was

TABLE II

Effect of Removing Spleen at Various Intervals after Intravenous Administration of Virus

Number of monkeys inoculated	Date of injection*	Date of splenectomy	Interval	Results
			days	
2	July 20	July 21	1	No infection
2	" 20	" 23	3	" "
2	" 20	" 25	5	" "

* 10 cc. 10 per cent virus-cord intravenously.

TABLE III

Effect of Removing Spleen at Various Intervals after Intrasplenic Administration of Virus

Monkey	Date of injection*	Date of splenectomy	Interval	Results
			days	
7	Apr. 24	Apr. 25	1	Poliomyelitis
8	" 16	" 17	1	No infection
9	" 16	" 19	3	" "
10	" 16	" 19	3	" "
11	" 16	" 21	5	" "
12	" 24	" 29	5	" "

* 2 cc. 10 per cent virus-cord intrasplenically.

accomplished by loosely suturing the abdominal muscles about the splenic pedicle and closing the skin over the spleen. Enough of the pedicle was brought out to make the subsequent splenectomy simple; the skin was incised to expose the spleen, the pedicle was clamped off, the spleen removed and bleeding vessels tied off or cauterized. Careful inspection for remnants of splenic tissue was made before closing the incision.

Six monkeys with exteriorized spleens were injected intrasplenically with 2 cc. 10 per cent virus suspension 2 weeks after operation. Splenectomy was done 1, 3 and 5 days after injection. In only one instance did infection occur; this was in a monkey splenectomized 24 hours after injection, paralysis appearing 6 days after the operation. We believe that infection in this animal was probably caused by leakage of the virus into the peritoneal cavity or subcutaneous tissues because: (*a*) doses of virus 5 times larger than the one used here uniformly failed to produce infection by the intravenous route, and (*b*) a large part of the inoculum, as will be shown later, is fixed by the spleen and so removed from the body when the spleen is removed. This experiment, therefore, did not demonstrate that splenectomy subsequent to intravenous or intrasplenic introduction of virus served to decrease resistance. This absence of increased susceptibility, however, may be due in major part to the removal of a large portion of the infectious agent from the body after its accumulation in the spleen.

Attempts to Infect Monkeys by Intrasplenic Injections of Virus

The experiments just described show that small doses of virus given intrasplenically are no more effective in producing poliomyelitis than are similar doses given intravenously. We were interested in determining the effects of repeated administrations of virus intrasplenically, since Brebner (4) and Schaeffer (10) state that many lethal intracerebral doses of virus can be given by this route without infecting the animal.

Seven monkeys with exteriorized spleens were given intrasplenic injections of virus at weekly intervals. 3 other animals were given only 2 injections with a 2 day interval between the inoculations. The results (Table IV) confirm the findings of Brebner and Schaeffer. Only 2 of the 10 animals succumbed to infection, and the possibility of virus leakage into the peritoneal cavity or subcutaneous tissues, or infection of nerve endings within the spleen, cannot be ruled out.

The administration of virus by this route leads to the appearance of humoral antibodies, 2 injections presumably being sufficient to stimulate their formation in amounts detectable by our usual serum neutralization technic (1.5 cc. of serum mixed with 0.5 cc. 1 per cent

virus, the entire amount being injected intracerebrally into test monkeys after incubation for 2 hours at 37°C. and overnight in the refrigerator).

Fixation of Virus by the Spleen

Luckhardt and Becht (11) found that antigen[2] injected intravenously accumulates in the spleen in considerable quantity. Topley (12), using *Bacterium paratyphosum* B, noted that the spleen re-

TABLE IV
Effect of Intrasplenic Inoculation of Poliomyelitis Virus

Monkey	Intrasplenic injection				Number of weekly injections	Results		Serum neutralization test*
13	2 cc. 10 per cent virus				1†	Poliomyelitis		0
14	1 " 20 "	"	"	"	3†	"		0
15	1 " 20 "	"	"	"	6	No infection		Positive
16	1 " 20 "	"	"	"	6	"	"	"
17	1 " 20 "	"	"	"	6	"	"	"
18	1 " 20 "	"	"	"	6	"	"	"
19	1 " 20 "	"	"	"	6	"	"	"
20	2 " 10 "	"	"	"	2‡	"	"	"
21	2 " 10 "	"	"	"	2‡	"	"	"
22	2 " 10 "	"	"	"	2‡	"	"	Negative

0 = not done.

* Serum obtained 4 weeks after injection of virus.

† Poliomyelitis before series carried further; monkey 13 became paralyzed 7th day after injection, monkey 14 on the 4th day after the 3rd injection.

‡ Injections given 2 days apart.

moves much of the antigen from the blood and is active in destroying it. Accumulation and destruction in the spleen of malarial parasites, organisms especially suitable for studies on cellular immunity, has been described in detail recently by Taliaferro and Cannon (13). Deutsch (14) observed that splenectomy performed before inoculation of bacterial antigen exerted little or no effect on subsequent antibody production, while splenectomy done 3 to 5 days after inoculation significantly depressed antibody formation.

[2] Goat's red blood cells, injected into dogs.

To determine the rôle of the spleen in fixing and disposing of the virus, 18 monkeys, including immune animals as controls, were injected intravenously or intrasplenically. Single injections of virus were given in every instance; 10 cc. of a 10 per cent virus suspension intravenously, or 2 cc. of the same suspension intrasplenically. On the 1st, 3rd and 5th days of the experiment the spleens were removed aseptically under ether or nembutal anesthesia. Immediately after removal the spleens were ground with 3 volumes of sterile saline and the suspension placed in the refrigerator for several hours to allow settling of large particles; 2 cc. of the supernatant fluid was injected intracerebrally into test monkeys.

TABLE V

Accumulation of Poliomyelitis Virus in the Spleen after Intravenous Injection

Spleen of monkey	History of monkey	Interval between inoculation and splenectomy	Test monkey*	Results in test monkeys
		days		
A	Normal	1	23	Poliomyelitis
B	"	1	24	"
C	Convalescent	1	25	No infection
D	"	1	26	" "
E	Immune	1	27	" "
F	Normal	3	28	" "
G	"	3	29	" "
H	"	5	30	" "
J	"	5	31	" "
K	Convalescent	5	32	" "
L	Immune	5	33	" "
M	"	5	34	" "

* Each animal received intracerebrally 2.0 cc. of a 25.0 per cent crude suspension of spleen in saline.

The suspensions of spleens removed from normal monkeys 24 hours after inoculation of virus into the blood or spleen contained virus demonstrable by inoculation tests, while spleens removed on the 3rd and 5th days did not (Tables V and VI). In no instance was virus detectable in the spleens of immune animals. No attempt was made to detect virus in the blood at the time of splenectomy, since Clark, Fraser and Amoss (15) have found that the virus persists in the blood for at least 3 days when large amounts (180 cc.) are administered

intravenously, but is detected only occasionally at the end of 24 hours when small amounts (10 cc.) are given.

Virus introduced into the blood stream of normal monkeys, then, rapidly accumulated in the spleen, persisted for at least 24 hours and disappeared by the 3rd day. The failure to detect virus in immune spleens is perhaps due to its neutralization by humoral antibodies or to rapid destruction by sensitized splenic tissue (16).

To determine the fate of the virus, we tested for the presence of neutralizing antibodies in the sera of monkeys splenectomized before and at intervals after intravenous injection of virus.

TABLE VI

Fixation and Destruction of Poliomyelitis Virus by the Spleen after Intrasplenic Injection

Spleen of monkey	History of monkey	Interval between inoculation and splenectomy	Test monkey*	Results in test monkeys
		days		
N	Normal	1	35	Poliomyelitis
P	"	1	36	"
Q	"	3	37	No infection
R	"	3	38	" "
S	"	5	39	" "
T	"	5	40	" "

* Each monkey received 2.0 cc. intracerebrally of a 25.0 per cent crude suspension of spleen in saline.

Eight non-splenectomized normal monkeys and 2 splenectomized normal monkeys were given a single injection of 10 cc. 10 per cent virus intravenously. The splenectomized monkeys and 2 of the intact animals served as controls; the remaining 6 animals were splenectomized at 1, 3 and 5 day intervals, as shown in Table V (monkeys A, B, F, G, H and J).

All the experimental animals survived without signs of infection; sera obtained 4 to 6 weeks after inoculation were tested for antiviral properties (Table VII), 1.5 cc. of serum being tested against 0.5 cc. 1.0 per cent virus. Table VII shows that neutralizing antibodies were present in the sera of both the non-splenectomized controls and the animals splenectomized prior to inoculation. Those animals

which were splenectomized subsequent to inoculation, however, failed to develop neutralizing antibodies.

Removal of the spleen 2 weeks prior to inoculation, therefore, did not qualitatively affect antibody formation. Splenectomy within 5 days after inoculation, however, seemed to suppress antibody formation. The simplest explanation appears to be that in the absence of the spleen other parts of the reticulo-endothelial system remove the

TABLE VII

*Effect of Splenectomy on Antibody Formation after Intravenous Administration of Virus**

Monkey	Treatment	Results		Serum neutralization test†
41	Splenectomized 14 days before inoculation	No infection		Positive
42	" 14 " " "	"	"	"
A	Splenectomized 1 day after inoculation	"	"	Negative
B	" 1 " " "	"	"	"
F	Splenectomized 3 days after inoculation	"	"	"
G	" 3 " " ' "	"	"	"
H	Splenectomized 5 days after inoculation	"	"	"
J	" 5 " " "	"	"	"
43	Non-splenectomized control	"	"	Positive
44	" " "	"	"	"

* Each animal received one dose of 10.0 cc. 10 per cent crude virus-cord suspension.

† Serum obtained 4 to 6 weeks after inoculation.

virus from the circulation; the spleen, when present, filters out an appreciable amount, as evidenced by the fact that spleens removed 24 hours after vascular inoculation contain active virus. Inasmuch as removal of the spleen at this time leads to a simultaneous removal of antigen, little or no antibody formation might be expected. The fact that spleens removed on the 3rd and 5th days contain no demonstrable virus, and the animals from which such spleens are removed fail, in contrast with control animals, to develop antiviral bodies,

suggests that little or no virus escapes from the spleen after its fixation there, and that it is destroyed *in situ*. These findings agree with those of Topley (12) and Deutsch (14), who used bacterial antigens.

Attempts to Detect Virus in the Spleens of Poliomyelitic Monkeys

The hyperplasia of lymphoid tissue which occurs throughout the body in human and experimental poliomyelitis has been interpreted by many authors as evidence of a systemic infection (7 *a*), a view supported by the claims of clinicians that patients react as to a generalized infection. The virus has not been detected in human blood (7 *a*) even when large amounts have been tested, but has been found occasionally in monkey blood (7 *a*) at the height of paralysis. Failure to isolate the virus more frequently may be due to the presence of neutralizing antibodies, which have been detected in the blood of monkeys 36 hours after the onset of paralysis (17) and in human cases during and even prior to paralysis (18), but since the virus displays such great affinity for nervous tissue, one would expect to find little outside the central nervous system. According to Fairbrother and Hurst (19), aside from the central nervous system the virus is found with regularity only in the tonsils and nasopharyngeal mucosa.

In the experiments described above, the virus, when administered intravenously or intrasplenically, was demonstrated in the spleen at the end of 24 hours, but not at 72 hours. Inability to detect the virus under these conditions, as well as in the blood of human cases, and of monkeys inoculated by other routes, may rest on its destruction by phagocytes or neutralization by antibodies. The latter possibility is of fundamental importance as well as of considerable interest. The simultaneous presence of virus and antibodies in the blood has been demonstrated in experimental vaccinia by Smith (20) and in a human case of yellow fever by Berry and Kitchen (21). Discussing such observations, Topley and Wilson (22) point out that "caution should be exercised in assuming that any tissue extract or body fluid is necessarily free from living virus because it is not infectious."

To gain information on these points we prepared extracts of spleens removed from 10 monkeys in the acute stage of poliomyelitis following intracerebral

inoculation of virus. Crude suspensions were made of 5 spleens by grinding the tissue with 3 volumes of saline and freezing and thawing 10 times in a carbon dioxide-alcohol mixture. After several hours in the refrigerator to allow coarse particles to settle out, 2.0 cc. of the supernatant fluid was injected intracerebrally into test monkeys. None of these animals showed any clinical evidence of infection. 2 were subsequently inoculated intracerebrally with 1.0 cc. of 1.0 per cent crude virus-cord suspension and succumbed to poliomyelitis. Another pair was sacrificed to examine the spinal cords, which in both instances were found to show no histological evidence of poliomyelitis.

The remaining 5 spleens were perfused with physiological salt solution until the perfusate was clear and the organ acquired a pale yellow to white color. The tissue was then ground with 3 volumes of saline, frozen and thawed 10 times in a carbon dioxide-alcohol mixture and injected intracerebrally (2.0 cc.). One test animal succumbed to poliomyelitis, paralysis appearing 15 days after inoculation of the splenic extract; the other 4 gave no evidence of infection. 2 died of intercurrent infection 1 and 3 months after inoculation and 2 were sacrificed at the end of 2 months observation. In 2 of these animals the histologic findings were negative, in one the findings were compatible with a diagnosis of poliomyelitis but were not typical of the experimental disease, and in one typical poliomyelitic changes were present.

The failure to demonstrate virus in crude suspensions, in contrast to extracts of the perfused organs, may depend on the concomitant presence of antibodies. To test this hypothesis, the following experiments were done.

Experiment 1.—The spleens of 2 normal and 2 immune monkeys were removed aseptically and ground with sterile saline to give a 25 per cent suspension. After the coarser particles had settled out, 1.5 cc. of the turbid supernatant was mixed with 0.5 cc. of a 1.0 per cent crude virus-cord suspension. The mixtures were incubated 2 hours at 37°C., and overnight in the refrigerator; after being warmed to body temperature, 2.0 cc. was injected intracerebrally into monkeys. Suspensions of immune spleens neutralized the virus, while both normal spleens failed to do so (Table VIII).

Experiment 2.—Spleens were removed aseptically from 9 monkeys (3 immune, 3 normal and 3 dying of poliomyelitis) and perfused with physiological salt solution until the perfusate was clear. At the end of this treatment the spleens presented a glistening, yellow, wax-like appearance. Microscopic sections showed the presence of a relatively small number of red blood cells and also that a large part of the pulp cells had been washed out. The perfused spleens were ground with saline to make a 25 per cent suspension, the supernatant being a yellowish, heavily opalescent fluid as compared with the deep brown-red of the crude suspensions. The extracts were frozen 10 times in a carbon dioxide-alcohol mix-

ture, passed through a Berkefeld N filter and the filtrates used in neutralization tests, which were done in the same manner as for the crude suspensions above. No neutralization of the virus occurred with any of the extracts (Table VIII).

TABLE VIII

Tests for Neutralizing Antibodies in Crude and in Perfused Extracts of Monkey Spleens

Test monkey	Spleen from	Intracerebral injection into test monkeys	Results in test monkeys
45	Immune monkey 1	2 0 cc. mixture of 1.5 cc. crude 25 per cent suspension of spleen plus 0.5 cc. 1 per cent virus-cord	No infection
46	" " 2		" "
47	Normal monkey 1		Poliomyelitis
48	" " 2		"
49	Poliomyelitic monkey 1*		Poliomyelitis
50	" " 2*		"
51	" " 3*		"
52	Normal monkey 1	2 0 cc. mixture of 1.5 cc. 25 per cent suspension of perfused spleen plus 0.5 cc. 1 per cent virus-cord	"
53	" " 2		"
54	" " 3		"
55	Immune monkey 1		"
56	" " 2		"
57	" " 3		"
58		2.0 cc. mixture of 1.5 cc. pooled convalescent monkey serum plus 0.5 cc. 1 per cent virus-cord	No infection
59		2.0 cc. mixture of 1.5 cc. pooled normal monkey serum plus 0.5 cc. 1 per cent virus-cord	Poliomyelitis

* Spleen removed at height of paralysis after intracerebral injection of virus.

These experiments show that it is possible to remove humoral antibodies from the spleen and suggest that our success in detecting virus in the perfused spleens was due to the removal of a neutralizing substance. If virus and neutralizing substances are present simultane-

ously in poliomyelitic spleens the virus might, if present in sufficiently high concentration, act as an antigen in stimulating the formation of poliocidal antibodies, since several authors (23) have reported a high incidence of immunity in monkeys following injections of neutralized mixtures of virus and serum.

Spleens were removed aseptically from monkeys at the height of paralysis following intracerebral inoculation of virus. 25 per cent suspensions in saline were prepared from each spleen and injected into 3 monkeys by various routes. Injections were given at the rate of 2 a week for 7 weeks, a total of 41.0 cc. being administered to each monkey. Sera obtained 6 weeks after the last injection failed to neutralize the test virus (1.5 cc. serum plus 0.5 cc. 1.0 per cent crude virus-cord suspension).

Considering the results obtained in the perfusion experiments, it is unlikely that all the spleens were devoid of virus. It appears more probable that the relatively large amount of inoculum contained too little virus to excite an appreciable antibody response. Although poliomyelitic spleens may contain amounts of virus infectious upon intracerebral inoculation it does not necessarily follow that this concentration is sufficient, in view of the known weak antigenic properties of the virus, to elicit any marked antibody response. This point has been emphasized recently by Kramer (23 d) who found that neutralized mixtures containing the equivalent of 1.5 gm. of virus-cord failed in many instances to produce sufficient antibodies to neutralize a small test dose of virus.

DISCUSSION

The necessity of using huge doses of virus to produce experimental poliomyelitis in *Macaca mulatta* by the intravenous route is well known and generally attributed to the difficulty encountered by the virus in reaching susceptible nerve tissue, a difficulty readily appreciated when it is considered that the nervous system is well isolated from other tissues (24). This insulating mechanism, however, is defective in at least one region, *viz.* the olfactory mucosa, where the endings of the first and thirteenth cranial nerves lie free. Lennette and Hudson (8) have shown that following intravenous administration of large doses the virus can be detected in nasal washings and that infection probably depends to a considerable degree on the amount

of virus excreted onto the nasal mucosa, a concept which finds support in the recent demonstration (25) of lesions in the olfactory bulbs of monkeys succumbing to infection by this route and in the ability of nasally instilled picric acid to prevent infection after vascular inoculation of the virus (26). While large amounts of virus accumulate in the spleen, and therefore should decrease the amount available for excretion onto the nasal mucosa, we were unable to detect any difference in susceptibility to infection, after repeated intravascular inoculation, between splenectomized and non-splenectomized animals. The possibility that the large doses of virus used (3 daily injections of 10 cc. 10 per cent crude virus-cord suspension) obscured any slight differences in susceptibility was considered, and the dosage decreased. A single injection of crude 10 per cent virus, while found not to be infective for a large group of non-splenectomized monkeys proved to be so for a small number of splenectomized animals. The results, while not conclusive due to the small number of splenectomized animals employed, suggest that some decrease in resistance to infection may occur when the animal is splenectomized prior to inoculation.

Splenectomy subsequent to intravenous or intrasplenic injection of virus did not appear to alter resistance to infection by these routes; this may be due, however, to simultaneous removal of much of the virus, which was found to accumulate rapidly in the spleen. Spleens removed from animals inoculated by either of the routes mentioned contained virus 24 hours after inoculation, but none after 3 or 5 days. The results of these experiments also suggest that splenectomy, with a simultaneous removal of a considerable portion of the antigen, interferes with antibody formation. None of 6 monkeys splenectomized after vascular inoculation developed neutralizing antibodies whereas the controls did so. Also, since no virus was detected in spleens removed 3 and 5 days after inoculation, and since the animals from which these spleens were obtained failed to develop poliocidal antibodies, we are inclined to believe that the virus is destroyed in the spleen.

Attempts to detect virus in the spleens of monkeys infected by routes other than the vascular were complicated by the presence of a neutralizing factor. Crude suspensions of poliomyelitic spleens failed to produce poliomyelitis on intracerebral injection, while similar

spleens after perfusion in one case caused frank paralysis and in two others lesions in the spinal cord. In one instance the lesions were typical of the experimental disease and in the other compatible with a diagnosis of poliomyelitis, although not absolutely typical.

An attempt to immunize test animals with poliomyelitic spleen tissue failed. The negative results presumably were due to a small content of virus rather than to the presence of neutralizing antibodies.

SUMMARY AND CONCLUSIONS

1. No difference in susceptibility to infection between splenectomized and non-splenectomized monkeys was observed when virus was injected intravenously in large amounts.

2. In a small series of animals, the results suggested that splenectomy decreased resistance to amounts of virus subinfective for non-splenectomized monkeys.

3. Splenectomy subsequent to intravenous or intrasplenic inoculation of amounts of virus subinfective for non-splenectomized monkeys did not render the animals susceptible.

4. Following vascular injection, the virus accumulates in the spleen, where it can be detected 24 hours after inoculation, but not at 3 or 5 days.

5. Evidence that splenectomy interferes with antibody formation is presented and discussed.

6. We were unable to demonstrate virus in crude extracts of poliomyelitic spleens but were successful with perfused spleen extracts. This may be due to removal of a neutralizing factor.

7. Attempts to immunize test animals with virus present in poliomyelitic spleens were negative.

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GLOBIN UTILIZATION BY THE ANEMIC DOG TO FORM NEW HEMOGLOBIN

By F. S. ROBSCHEIT-ROBBINS, Ph.D., and G. H. WHIPPLE, M.D.

(From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

In all work relating to hemoglobin one unconsciously has in mind the pigment radicle as being of prime importance because it is a conspicuous factor due to its color and its relation to other body pigments (bile pigment, urobilin and related substances). The pyrrol aggregate—a compound of four pyrrol rings—is common to bile pigment and to the hemoglobin pigment radicle.

Globin by contrast has been little studied but its importance probably is in proportion to its size and it makes up about 95 per cent of the hemoglobin molecule. It is antigenic (4) and its chemical make-up has been studied but we know very little about its manufacture within the body and what happens when it is broken down in body metabolism.

One can readily show (6) that the anemic dog can use goose and sheep hemoglobin just as completely as dog hemoglobin when given intravenously, and if we give 50 gm. of either foreign hemoglobin we confidently expect to remove from the standardized anemic dog 45 to 50 gm. new dog hemoglobin to maintain the anemia level a constant. In a great number of experiments the return averages approximately 100 per cent—that is the dog conserves completely hemoglobin given by vein whether the hemoglobin derives from the dog, goose or sheep. As hemoglobin is species specific it is obvious that the foreign hemoglobin must be broken down to some types of intermediates before it is recast into the new dog hemoglobin.

Other experiments (3) indicate the importance of the *globin fraction* in contrast to the pigment radicle. If to an anemic *bile fistula* dog we give by vein 50 gm. of dog or sheep hemoglobin, we observe a return in new hemoglobin of 45 to 50 gm. just as in the anemic non-

fistula dog. But we observe also a great increase in bile pigment eliminated and can account for 60 to 100 per cent of the pigment radicle contained in the injected hemoglobin. Whatever our explanation may be the body obviously can produce a large excess of the pigment radicle promptly whether it is eliminated in the urine as bile pigment or incorporated in the new formed hemoglobin. This experiment would point to the *globin* as the limiting factor in new hemoglobin production and would indicate the need for a detailed study of all aspects of globin utilization within the body.

We should keep in mind the fact that the anemic fasting dog when given iron by mouth or vein can produce 100 gm. or more of new hemoglobin. The globin and pigment radicles obviously are formed from the proteins within the body (tissue, parenchyma or plasma proteins) (2). This effort by the fasting anemic dog cannot be long continued but the proof is conclusive that in this emergency (anemia) the body proteins can contribute to the formation of new hemoglobin and its contained globin.

Our interest in the reaction of the anemic dog to *globin* given by mouth or by vein needs no further comment. The tables below indicate that the standard anemic dog in order to form hemoglobin utilizes *globin* by mouth to good advantage. Globin given orally averages 30 per cent utilization in contrast to a 10 to 15 per cent utilization of hemoglobin given by mouth.

Globin given by vein does not give a uniform reaction as is true for hemoglobin given by vein. The reasons for this difference in reaction may be subject to debate. Hemoglobin is a naturally occurring substance in the circulation as red cells disintegrate from normal wear and tear. Globin as we use it to introduce into the circulation is probably a foreign substance as it is often toxic in small doses and always toxic in large doses. In other words the breakdown of hemoglobin within the body produces a slightly different substance than the globin produced by acid acetone precipitation in the method used below.

Globin prepared from dog or horse hemoglobin given by vein can be well utilized in certain experiments to form new hemoglobin in the standard anemia dog (Tables 1 to 3). The surplus hemoglobin appears usually in the period following the injection of globin and

there may actually be some inhibition of hemoglobin production during the period of injection. The intoxication (severe, mild or non-recognizable) associated with globin injection is probably responsible for this delay and may explain the negative experiments. We have shown elsewhere (5) that the intoxication associated with a sterile abscess or endometritis will temporarily interfere with the internal metabolism responsible for new hemoglobin production in anemia.

Methods

All method details relating to the general anemia program have been described quite recently (7) and need not be repeated. Globin was prepared from horse and dog hemoglobin according to the method of Anson and Mirsky (1) which is essentially a precipitation by a solution of acetone and hydrochloric acid.

We are indebted to Dr. A. E. Mirsky for a careful explanation of this method and a generous supply of the globin which was used in preliminary experiments. The globin as used is a grey-white powder readily soluble in water with dextrose for intravenous use. It is probably digested with relative ease when fed with the standard salmon bread.

The artificial *digest* used in Table 1 and given by vein was prepared as follows: Horse globin 60 gm. in 3000 cc. 0.2 per cent HCl placed in incubator for 4 days. Solution was neutralized to phenolphthalein with NaOH and 2 gm. each of trypsin and erepsin was added. Material kept in incubator for 4 weeks. Reaction was kept alkaline to phenolphthalein. Hydrolysate was filtered and concentrated to 800 cc. under diminished pressure at about 40°C. Concentrated NaOH was added and NH₃ was removed by distillation under diminished pressure at temperature of 40°. Hydrolysate was immediately treated with 10 per cent HCl until reaction was just alkaline to litmus. Solution was sterilized in autoclave for 30 min. at 15 pounds pressure and kept at ice box temperature. No precipitation on standing. This hydrolysate was prepared under the supervision of Dr. F. S. Daft.

EXPERIMENTAL OBSERVATIONS

Unfortunately solutions of globin given by vein are somewhat toxic and at times may be very toxic. Small doses of globin must be given (1 gm. usually dissolved in 10 per cent dextrose) and in spite of every precaution and slow injection into the jugular we may observe at times clinical disturbances (fall in blood pressure, chill, vomiting or prostration) and on rare occasions moderately severe shock. Dog globin appears to be a little less toxic than horse globin. As the

standardized dogs are valuable we hesitated to use larger doses for fear of severe or lethal intoxication.

Table 1 (dog 27-236) shows three satisfactory experiments on the same dog at different times. In the first experiment *horse globin* is given *intravenously* over a period of 3 weeks amounting to 21 gm. in all. The dog was not clinically disturbed and ate all of the basal ration. There was a rise in hemoglobin level during the period of injection but no bleeding was done at this time. The new hemoglobin production was completed by the end of the 2nd control week. We note a net hemoglobin production above the basal output of 24 gm. new hemoglobin as a result of injecting 21 gm. globin.

Globin digest (Table 1) shows a similar reaction. When we give intravenously a tryptic digest derived from 18.3 gm. horse globin during a 2 weeks period we note a production of 25 gm. new hemoglobin above the base line output. There is a slight decrease in basal ration consumption and one might expect a lessened output of hemoglobin because of this slight intoxication (5). The fact that there is a slight excess of hemoglobin production above the globin digest intake we are not inclined to stress as it comes within the limits of physiological variables.

In general the tables below show good evidence that the anemic dog can use dog or horse globin given by vein to produce much new hemoglobin. The reaction often may be delayed and occasionally absent but we believe this is to be explained in large part as due to the toxic reaction of the dog to the artificially prepared globin solution.

Clinical Experimental Histories—Table 1.

Dog 27-236. Born Feb., 1928. Uneventful anemia history Apr., 1930, to Aug., 1935.

June 10, 1931. Horse globin 0.66 gm. to 1 gm. injected intravenously daily in 75 to 150 cc. 10 per cent dextrose solution. Material injected very slowly. After 11 doses dog showed moderate diarrhea and slight vomiting for 1 day. No further reaction.

Apr. 12, 1932. Globin digest by vein beginning with 10 cc. of the digest or the equivalent of 0.5 gm. horse globin plus 40 cc. 10 per cent dextrose. 3 hours after injection of first dose dog vomited. Dose was increased thereafter from 0.75 gm. equivalent to 2 gm. equivalent daily with from 40 to 100 cc. 10 per cent dextrose solution. After first 2 gm. equivalent dose dog left 70 per cent food for 1 day.

TABLE 1

Globin (Horse) Given by Vein and by Mouth
A Globin Digest also Utilized by Dog to Form Hemoglobin

Dog 27-236. Bull, male, adult.

Diet periods 1 wk. each	Food consumed	Weight	Plasma volume	R.B.C.	Blood Hb. level	Hb. removed, bled
Food, gm. per day	*per cent*	*kg.*	*cc.*	*mil.*	*per cent*	*gm.*
Bread 375, salm. 100, Kl. 30	100	13.4	894	4.6	48	1.3
*Globin 1 intrav., br. 375, salm. 100, Kl. 30	100	13.2	822	6.1	68	2.9
Globin 1 intrav., br. 375, salm. 100, Kl. 30	100	13 4	1028	6.9	67	2.9
Globin 1 intrav., br. 375, salm. 100, Kl. 30	100	13.4	825	5.7	61	3.6
Bread 375, salm. 100, Kl. 30	100	13.3	794	5.3	63	28.2
Bread 375, salm. 100, Kl. 30	100	13.2	734	5.3	41	40.6
Bread 375, salm. 100, Kl. 30	100	13.1	782	4 0	41	1.2
Basal output 8 gm. Hb. per wk. Total net Hb. output 24 gm. Total Hb. = 79.4						
Bread 400, salm. 100, Kl. 40	100	16 1	934	5.4	48	1.3
†Globin digest intrav., br. 400, salm. 100, Kl. 40	91	15 6	855	6 4	55	1.6
Globin digest intrav., br. 400, salm. 100, Kl. 40	91	16 0	863	6.7	59	14.6
Bread 350, salm. 150, Kl. 50	90	15 9	848	6.8	51	26.2
Bread 400, salm. 150, Kl. 50	100	16.0	889	5.7	46	13.7
Bread 400, salm. 100, Kl. 40	100	16 1	894	5 6	45	12.2
Basal output 8 gm. Hb. per wk. Total net Hb. output 25 gm. Total Hb. = 68.3						
Bread 375, salm. 125, Kl. 50	100	16 0	938	4.7	44	1.2
Globin 6, br. 375, salm. 125, Kl. 50	100	16 3	893	5 2	51	16.5
Globin 6, br. 375, salm. 125, Kl. 50	100	16 6	936	5 9	38	34.7
Bread 375, salm. 100, Kl. 40	100	16 4	976	4.3	38	1.8
Bread 400, salm. 100, Kl. 40	100	16 8	1000	5.2	44	1.2
Basal output 5 gm. Hb. per wk. Total net Hb. output 34 gm. Total Hb. = 54.2						

Salm. = commercial canned salmon.
Kl. = Klim, a commercial skimmed milk powder.
* Total globin given 21 gm.
† Total globin equivalent 18.3 gm.

No further unfavorable reaction.   Total amount given equivalent to 18.3 gm. horse globin.

Sept. 30, 1933.   Globin feeding begun with daily dose of 6 gm. mixed with small amount of bread and fed prior to general feeding.

Globin feeding (Table 1) in the same dog gives about a 40 per cent return of new hemoglobin.   The dog was fed 6 gm. horse globin a day for 2 weeks—a total of 84 gm. and produced a net hemoglobin output of 34 gm. above the base line average.   This dog gave a very prompt response to the globin feeding and most of the new formed hemoglobin appeared during the 2 weeks of globin feeding.   This represents a maximal response to protein material given by mouth and exceeds considerably the ratio of *liver protein* intake to new hemoglobin output—approximately 8 to 1 or a 13 per cent utilization, if we leave out of consideration the iron contained in the liver.

Clinical Experimental Histories—Table 2.

Dog 24-59.   Born 1922.   Uneventful anemia history 1923 to 1932.

Apr. 28, 1931.   *Globin* injection by vein 1 gm. dissolved in 150 cc. 10 per cent dextrose solution.   Following the first injection there was a chill but no obvious clinical reaction to subsequent injections.

Dog 27-238.   Born Feb., 1927.   Continuous anemia history Nov., 1928, to date.   Diet at no time contained potent animal protein substances in effort to produce dietary anemia.   Experiments consisted of testing vegetables, minerals, drugs and amino acids.

Apr., 1933.   One dose of horse globin, 1 gm. in 10 per cent dextrose by vein was followed by shock.   Experiment discontinued.

Nov. 18, 1933.   Feeding experiment of horse globin, 7.5 gm. daily added to basal bread ration.   (See Table 5.)

Feb., 1936.   Horse globin by vein, first dose 0.25 gm. in 10 per cent dextrose. Dose gradually increased to 1 gm. which was followed by slight reaction.   With dose of 1.25 gm. marked reaction.   Dog vomited 10 min. after injection, pulse weak, cyanosis, slight diarrhea.   This occurred after 6 doses had been given.

Mar. 9, 1936.   Slight shock following 1.25 gm. horse globin.   Food consumption poor.   Horse globin decreased to 0.75 gm. and later again increased to 1 gm. Considerable reaction following 2nd dose of 1 gm.   Dosage decreased to 0.50 gm. and no more than 0.75 gm. given until experiment was complete, a total of 25 gm. horse globin.

Globin given by vein is utilized to form new hemoglobin as seen in Table 2, dog 24-59.   This experiment in some respects is different

TABLE 2

Globin (Horse) Given by Vein

Positive and Negative Experiments

Diet periods 1 wk. each	Food consumed	Weight	Plasma volume	R.B.C.	Blood Hb. level	Hb. removed, bled
Dog 24-59. Bull, male, adult						
Food, gm. per day						
Bread 450, salm. 75, Kl. 30	per cent	kg.	cc.	mil.	per cent	gm.
	100	16.9	1073	4.8	51	1.5
*Globin 1.0, br. 450, salm. 75, Kl. 30	100	17.3	1079	5.5	53	14.5
Globin 1.0, br. 450, salm. 75, Kl. 30	100	17 7	1124	4.9	48	10 4
Globin 1.0, br. 400, salm. 75, Kl. 30	100	17 8	1164	5 7	48	9.7
Globin 0.6, br. 400, salm. 75, Kl. 30	100	17 9	1174	4 8	49	1 3
Bread 450, salm. 75, Kl. 40	100	17 0	1094	5.7	53	15.6
Bread 450, salm. 75, Kl. 40	100	16 6	1054	5.7	42	25 7
Bread 450, salm. 75, Kl. 40	100	16 6	1085	4 6	49	12.9
Bread 450, salm. 75, Kl. 40	100	16 9	1127	5 6	48	26 8
Basal output 10 gm. Hb. per wk. Total net Hb. output 34 gm. Total Hb. = 116.9						
Dog 27-238. Coach, female, adult						
Bread 350, salm. 75, Kl. 20	100	16 8	974	4 5	47	1 3
*Globin 0.9, br. 350, salm. 75, Kl. 20	99	16 9	939	5.5	54	1.5
Globin 0.8, br. 350, salm. 75, Kl. 20	61	16 6	807	5 3	58	11.6
Globin 0.7, br. 350, salm. 75, Kl. 20	85	16 5	805	4 5	52	12.6
Globin 0.5, br. 350, salm. 75, Kl. 20	81	16 5	970	—	41	1 4
Globin 0.7, br. 350, salm. 75, Kl. 20	94	16 7	867	4.5	47	10 4
Bread 275, salm. 20, Kl. 20	84	16 6	949	4.1	47	1.4
Bread 250, salm. 20, Kl. 20	85	16 2	839	5 2	56	15.5
Bread 200, salm. 20, Kl. 20	91	15 9	871	6 0	44	24 4
Bread 275, salm. 20, Kl. 20	95	16 1	976	4 0	53	4 6
Bread 225, salm. 20, Kl. 20	78	16 0	953	4 4	47	1 4
Basal output 9 gm. Hb. per wk. Total net Hb. output 0. Total Hb. = 84.8						

* Total globin given 25 gm.

from a similar experiment given in Table 1. The total globin given was 25 gm. but the injections were spread over 4 weeks and consequently the control after period was likewise 4 weeks. There was no increase in the hemoglobin production (actually a slight decrease)

during the period of injection but the new hemoglobin production appears in the subsequent control weeks. It may not be fair to include the hemoglobin output during the 4th control week and under such circumstances the net hemoglobin output would be 17 gm. instead of 34 gm. as given. At any rate the true hemoglobin return from the injected globin will approximate 100 per cent if we assume that the true net hemoglobin figure is somewhere between 17 and 34 gm. hemoglobin. The toxic influence of the injected globin solution *delayed* the hemoglobin regeneration but did not prevent the body from utilizing the globin material in subsequent weeks.

Table 2, dog 27-238, shows a negative response to globin injections. There is no excess of hemoglobin produced above the basal level and there is some inhibition of hemoglobin production during the prolonged injection period. This dog (see Clinical experimental history 27-238) was quite sensitive to globin injections which produced clinical shock on several occasions and caused lack of appetite. We believe the lack of response to the globin injection with new hemoglobin production to be due to this obvious intoxication. This dog was given one dose of horse globin in 1933 or 3 years before this experiment and at that time the dog was intoxicated to such a degree that the experiment was discontinued. Whether this dose made the dog even more susceptible to subsequent doses we cannot say.

Clinical Experimental Histories—Table 3.

Dog 33-14. Born Nov., 1932. Uneventful anemia history Dec., 1933, to date.
Mar. 8, 1937. Dog globin by vein beginning with 0.75 gm. in 10 per cent dextrose. Dose gradually increased to 2 gm. daily. Some reaction to 2 gm. dose—rapid pulse, considerable coughing. Dog left food for 2 days. Globin omitted for 2 days. Subsequently 0.50 gm. globin caused similar toxic reaction. Globin omitted for 1 day. Globin then injected beginning with 0.50 gm. and gradually increasing to 1.75 gm. daily. A slight reaction to 1.75 gm. was noted. Globin 1.25 gm. for the remaining injection period. No further clinical disturbance. Dog in good condition at end of experiment.

Dog 30-117. Born Sept., 1930. Uneventful anemia history Oct., 1931, to date.
May, 1936. *Dog* globin by vein beginning with 0.25 gm. in 10 per cent dextrose. Dose gradually increased to 2 gm. daily. No reaction at any time. Food consumption 100 per cent throughout experiment. No hemolysis 2 hours after injection of globin.

TABLE 3
Globin (Dog) Given by Vein
Positive and Negative Experiments

Diet periods 1 wk. each	Food consumed	Weight	Plasma volume	R.B.C.	Blood Hb. level	Hb. removed, bled
Dog 33-14. Coach, female, adult						
Food, gm. per day	*per cent*	*kg.*	*cc.*	*mil.*	*per cent*	*gm.*
Bread 350, salm. 75, Kl. 20	100	14.1	803	4.6	44	1 2
*Globin 0.8, br. 350, salm. 100, Kl. 20	100	14.0	672	5.0	53	1.5
Globin 0.5, br. 200, salm. 150, Kl. 20	75	13.7	748	5.5	45	20.3
Globin 1.0, br. 225, salm. 125, Kl. 20	100	13.6	763	·4.6	45	1.4
Globin 0.9, br. 225, salm. 125, Kl. 20	100	13 6	812	4.4	44	1.3
Globin 0.9, br. 225, salm. 125, Kl. 20	100	13.4	777	4.7	48	1.4
Bread 250, salm. 100, Kl. 20	100	13.4	745	4.9	50	25.8
Bread 250, salm. 100, Kl. 20	100	13.4	777	4.7	48	1.4
Bread 250, salm. 100, Kl. 20	100	13.4	653	6.0	49	35.3
Bread 250, salm. 100, Kl. 20	100	13.7	806	4.5	45	12.9
Bread 250, salm. 100, Kl. 20	100	13 3	794	5.1	47	24.6
Bread 250, salm. 100, Kl. 20	100	13.4	722	4.5	47	1.4

Basal output 9 gm. Hb. per week.   Total net Hb. output 25.0 gm.   Total Hb. = 127.3

Diet periods 1 wk. each	Food consumed	Weight	Plasma volume	R.B.C.	Blood Hb. level	Hb. removed, bled
Dog 30-117. Coach, male, adult						
Bread 450, salm. 75, Kl. 20	100	15 8	970	4 4	43	1 2
†Globin 0.3, br. 450, salm. 75, Kl. 20	100	16.0	886	5.9	44	22.4
Globin 1.1, br. 450, salm. 75, Kl. 20	100	16 1	944	4 1	39	1.2
Globin 1.6, br. 450, salm. 75, Kl. 20	100	16 0	970	4.1	46	1.3
Globin 1.1, br. 450, salm. 75, Kl. 20	100	16.4	1006	—	36	1.0
Bread 450, salm. 50, Kl. 20	100	16.6	1018	4.5	56	12.2
Bread 450, salm. 50, Kl. 20	100	16.5	1000	5.9	48	20.4
Bread 450, salm. 50, Kl. 20	100	16 4	1025	5 7	43	29.6
Bread 450, salm. 50, Kl. 20	100	16 4	1010	4.1	42	14.5
Bread 450, salm. 50, Kl. 20	100	16 4	1022	4 0	42	1 2

Basal output 14 gm. Hb. per wk.   Total net Hb. output 0.   Total Hb. = 103.8

* Total globin given 28.3 gm.
† Total globin given 29 gm.

Table 3, dog 33-14, shows a complete experiment in which 28.3 gm. of globin are given intravenously during a 5 week interval and about 25 gm. of hemoglobin are produced over and above the control base line of 9 gm. hemoglobin per week. There is little hemoglobin produced during the 5 week injection period, distinctly less than the control output and the surplus hemoglobin all appears in the 5 week after period. This reaction is very like that recorded (5) for an endometritis. The globin injections caused some clinical disturbance and only small doses could be given. There was some lack of appetite in the 2nd week of globin injection. We believe this intoxication due to globin injections is responsible for the inhibition of hemoglobin production during the injection period but the building materials are not lost so that a surplus hemoglobin production appears when globin injections are discontinued.

Table 3, dog 30-117, shows a complete experiment in which the globin injections caused inhibition of hemoglobin production in the 4 week injection period but there was no surplus produced in the after period of 5 weeks. This dog showed little if any clinical evidence of intoxication and its appetite was not impaired. The effect of this subclinical intoxication is obvious in the injection period and it seems safe to assume some disturbance due to the globin injections which interfered with the normal mechanism of hemoglobin production. This dog has a high basal hemoglobin output of 14 gm. per week so that the inhibition of hemoglobin construction during the 4 weeks of injection amounts to more than 30 gm. hemoglobin.

Clinical Experimental Histories—Table 4.

Dog 35-1. Born Nov., 1934. Uneventful anemia history July, 1936, to date. Mar. 9, 1937. Dog globin by vein beginning with 0.25 gm. in 10 per cent dextrose. Dose gradually increased to 2.25 gm. daily. With that amount, slight clinical reaction resulted—rapid pulse and coughing. Dog left all food next day. No globin given for 2 days. Injections of globin again started with dose of 0.50 gm. gradually increased to 1.75 gm. without any further disturbance during rest of experimental period. Dog in good condition at end of experiment.

Table 4, dog 35-1, gives the details of a very interesting experiment. The globin injections caused no significant clinical disturbance and a large total of 31 gm. were given during 4 weeks. There is no

evidence of inhibition of hemoglobin production as the hemoglobin surplus is 59 gm. over and above the base line output of 6 gm. hemoglobin per week. In fact there is a surplus of 28 gm. hemoglobin not explained. We are inclined to this explanation. It is well known in pharmacology that some drugs are destructive or injurious in large doses but stimulating in small doses. This dose of globin in this dog may have been of just the proper amount to cause stimulation rather than inhibition.

TABLE 4

Globin (Dog) Given by Vein—31 Gm.

Hemoglobin Surplus Is 59 Gm.

Dog 35-1. Bull, male, adult.

Diet periods 1 wk. each	Food consumed	Weight	Plasma volume	R.B.C.	Blood Hb. level	Hb. removed, bled
Food, gm. per day	*per cent*	*kg.*	*cc.*	*mil.*	*per cent*	*gm.*
Bread 450, salm. 75, Kl. 20	100	19.5	1147	5.7	47	1.3
Globin 1.1, br. 450, salm. 50, Kl. 20	86	19.3	1152	6.1	55	1.6
Globin 0.6, br. 250, salm. 150, Kl. 20	92	18.5	1085	6.0	53	24.2
Globin 1.3, br. 350, salm. 150, Kl. 20	100	18.6	1142	5.5	38	19.3
Globin 1.5, br. 350, salm. 150, Kl. 20	100	18.8	1210	4 3	38	1.5
Bread 400, salm. 75, Kl. 20	100	18.7	1223	4.8	46	1.4
Bread 400, salm. 75, Kl. 20	100	18.7	1068	5.1	49	12.3
Bread 400, salm. 75, Kl. 20	100	18 9	1096	6.3	36	34.8
Bread 400, salm. 75, Kl. 20	100	18.8	1213	5.0	46	1.4
Bread 400, salm. 75, Kl. 20	100	18.9	1033	5.9	47	25.2
Bread 400, salm. 75, Kl. 20	100	18.6	1124	5.0	43	1.4

Basal output 6 gm. Hb. per week. Total net Hb. output 59 gm. Total Hb. = 123.1

Clinical Experimental Histories—Table 5.

Dog 23-1. Born 1922. Uneventful anemia history, Oct., 1924, to Mar., 1936. Dec., 1933. Horse globin feeding experiment for 2 weeks with daily dose of 7.5 gm. added to the basal salmon bread ration.

Dog 30-121. Born Sept., 1930. Uneventful anemia history, Oct., 1931, to date.

Mar. 3, 1936. Globin feeding experiment for 2 weeks with daily dose of 7.5 gm. added to basal salmon bread ration.

TABLE 5

Globin (Horse) Given by Mouth

Approximately 30 Per Cent Utilization

Diet periods 1 wk. each	Food con- sumed	Weight	Plasma volume	R.B.C.	Blood Hb. level	Hb. re- moved, bled
Dog 27-238. Coach, female, adult						
Food, gm. per day	*per cent*	*kg.*	*cc.*	*mil.*	*per cent*	*gm.*
Bread 350, salm. 125, Kl. 50	100	14 1	910	—	44	1.2
Globin 7.5, br. 375, salm. 100, Kl. 40	100	14.4	847	4.2	40	13.0
Globin 7.5, br. 375, salm. 100, Kl. 40	100	14.7	862	5 0	45	15.4
Bread 375, salm. 100, Kl. 40	100	14.8	868	4.5	48	1.4
Bread 375, salm. 100, Kl. 40	100	14 8	820	6.1	55	37.2
Bread 375, salm. 100, Kl. 40	100	15.1	902	4.7	44	12.9

Basal output 8 gm. Hb. per week.   Total net Hb. output 40 gm.   Total Hb. = 79.9

Dog 23-1. Bull, male, adult						
Bread 375, salm. 100, Kl. 40	100	17 9	1099	3.9	39	1.1
Globin 7.5, br. 375, salm. 100, Kl. 40	100	17.8	1063	5.1	51	12.6
Globin 7.5, br. 375, salm. 100, Kl. 40	100	18.0	1125	4.3	45	1.3
Bread 375, salm. 100, Kl. 40	100	17.7	1023	5.3	48	24.7
Bread 400, salm. 100, Kl. 40	93	17.7	1086	4.7	42	11.1
Bread 375, salm. 150, Kl. 50	60	17.0	1030	4.3	42	1.2

Basal output 8 gm. Hb. per week.   Total net Hb. output 14 gm.   Total Hb. = 50.9

Dog 30-121. Coach, male, adult						
Bread 450, salmon 50, Kl. 20	100	14.5	878	4.4	43	1.2
Globin 7.5, br. 450, salm. 50, Kl. 20	100	14.7	934	5.3	44	13.2
Globin 7.5, br. 450, salm. 50, Kl. 20	100	14.7	905	5.0	52	14.4
Bread 450, salm. 50, Kl. 20	100	14.9	943	5.8	45	24.6
Bread 450, salm. 50, Kl. 20	100	14.7	904	5.3	50	13.9
Bread 450, salm. 50, Kl. 20	100	14.9	914	4.6	48	1.3

Basal output 9 gm. Hb. per week.   Total net Hb. output 27 gm.   Total Hb. = 67.4

Table 5 shows three more experiments with horse *globin feeding*. The first experiment (dog 27-238) is practically a replica of that given in Table 1. A slightly larger amount of globin is fed (total 105 gm. during the 2 weeks period) and a net hemoglobin output of 40 gm. is recorded. The ratio of intake of globin to new hemoglobin production is 105 to 40 or a 39 per cent utilization of this protein to form hemoglobin. This is practically identical to the 40 per cent utilization given in Table 1 and represents maximal utilization for protein given by mouth.

The second experiment (Table 5, dog 23-1) shows a much lower level of protein utilization. With 105 gm. globin given by mouth the net hemoglobin production is only 14 gm.—a 13 per cent utilization. This is about the same as the utilization of liver protein for hemoglobin regeneration—a ratio of 8 gm. intake to 1 gm. hemoglobin output.

The third experiment (Table 5, dog 30-121) shows an intermediate level of protein utilization. Given 105 gm. globin by mouth, the dog produces a surplus of 27 gm. new hemoglobin—a 26 per cent utilization of this protein. The average of all four globin feeding experiments is a 30 per cent utilization—that is given 100 gm. globin by mouth we should expect a net surplus of new hemoglobin amounting to 30 gm.

SUMMARY

It has been shown that the standard anemic dog can use sheep, goose or dog hemoglobin when given by vein and return quantitatively its equivalent as new dog hemoglobin within the red cells.

Globin at times can be used when given by vein with a quantitative return of new hemoglobin in red cells in these same anemic dogs. Again the administration of globin by vein will inhibit the expected hemoglobin formation; and we believe the toxic effect of the globin is responsible. A digest of globin may be used by the anemic dog to form new hemoglobin. Globin from both horse and dog have been tested and seem to react in identical fashion.

The globin radicle of hemoglobin appears to be an important limiting factor in abundant hemoglobin building in this type of anemia due to blood loss.

Globin fed by mouth is well utilized to form new hemoglobin and we may record a 30 to 40 per cent utilization or a return of 30 to 40 gm. new hemoglobin from the feeding of 100 gm. globin. This is to be compared with the utilization of liver protein—an average return of 13 gm. new hemoglobin for the feeding of 100 gm. liver protein.

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PANMYELOPHTHISIS WITH HEMORRHAGIC MANIFES-
TATIONS IN RATS ON A NUTRITIONAL BASIS*

By PAUL GYÖRGY, M.D., HARRY GOLDBLATT, M.D., FRANKLIN R.
MILLER, M.D., AND ROBERT P. FULTON, M.D.

*(From the Babies and Childrens Hospital, the Institute of Pathology, and the
Departments of Pediatrics, Pathology, and Medicine, School of Medicine,
Western Reserve University, Cleveland)*

INTRODUCTION

Vitamin B_6 is that part of the vitamin B_2 complex which is respon-
sible for the cure of the specific dermatitis ("rat acrodynia"[1]) developed
by young rats fed a vitamin B free diet supplemented with vitamin
B_1 and lactoflavin (3).

A good and potent source of vitamin B_6, free from lactoflavin, is
Peters' eluate (4), which is obtained essentially by elution of a char-
coal adsorbate of an aqueous yeast extract with acid alcohol.

Lepkovsky, Jukes and Krause (5) have shown that rats need, in
addition to vitamin B_6 and lactoflavin, a third, mainly growth-
activating fraction of the vitamin B_2 complex. This is also called the
"filtrate factor" (6), owing to the outstanding chemical properties
revealed during the course of preliminary attempts at its purification.

In the synthetic diet first used (3) for the production of rat acro-
dynia, rice starch was employed as the source of food carbohydrate.
When the experiments for purification of vitamin B_6, preliminary re-
sults of which have been reported (7), were resumed, rice starch was
replaced in the diet first by corn starch, then by cane sugar. This
substitution was made after a specimen of rice starch had proved toxic
for rats and after it was found that corn starch, at least the brand

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[1] The term acrodynia which appears throughout this article refers to these
skin manifestations in rats fed a diet deficient in vitamin B_6 (2).

used, prevented or delayed the appearance of rat acrodynia, in confirmation of relevant observations by Richardson and Hogan (8).

Rats kept on a diet of this kind, deficient in vitamin B_6 and containing sugar, developed acrodynia in from 6 to 20 weeks. Treatment with purified, less crude B_6 preparations was in many cases followed not by complete recovery but by a very acute, fatal anemia and a great variety of symptoms of a concomitant hemorrhagic diathesis. Medication with more complex B_6 preparations, such as Peters' eluate, or with certain foodstuffs, such as milk, liver and yeast, assured final cure of the B_6 deficiency disease without the complication of abnormal hematopoiesis.

In several instances this peculiar hemorrhagic disease has been observed by us in rats when there was no change in the experimental conditions. On further study it became evident that this disease, which at first gave the impression of being purely accidental, was a distinct pathologic entity, with a well defined etiologic basis. We were able to show that one constituent of the vitamin B_2 complex plays, apparently, an important part in primary blood cell production.

EXPERIMENTAL DATA

Rats, mostly albino, 21 days of age, weighing not more than 35 gm., were placed on the following diet.

	per cent
Casein[2]	18
Cane sugar	68
Melted butter fat	9
Salt mixture (McCollum 185)	4
Cod liver oil	1

Each rat in an experiment received daily, in addition to the diet, 3 pigeon units of a highly purified vitamin B_1 preparation employed in previous studies (2) and 10γ of crystalline natural lactoflavin, which was replaced in later experiments by crystalline synthetic lactoflavin.[3]

Animals with symptoms typical of acrodynia were treated with vitamin B_6 preparations or with foodstuffs containing vitamin B_6.

In those animals in which anemia and hemorrhagic manifestations followed the successful treatment of vitamin B_6 deficiency, the first sign of the impending new disease consisted in renewed suspension of growth and in reduced food intake.

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[2] Purified casein chiefly was used, but the results were duplicated in some experiments with Merck's commercial casein.

[3] Vitamin B_1 and lactoflavin were furnished through the courtesy of Winthrop Chemical Company, Inc., New York.

In untreated animals, anemia and hemorrhagic symptoms were often preceded by spontaneous cure of a very mild acrodynia that usually started not more than 1 to 2 weeks earlier, and by further pronounced anorexia.   Only exceptionally did we observe rats in which the hemorrhagic disease came about without such premonitory symptoms and in particular without preceding acrodynia.

Onset of the anemia was easily recognized in the albino rats by comparing the color of their eyes with that of normal rats.   A drop of 10 to 20 per cent in the hemoglobin content of the blood could thus be estimated without difficulty.   The hemorrhagic diathesis was indicated by variable signs.   One of the most common symptoms was nosebleed.   Usually this was not profuse and manifested itself only in slight bloody discharge which soon dried up.   In albino animals the bleeding stained mostly the fur around the nose and, owing to repeated rubbing, also the forepaws.   In other cases the tear fluid was hemorrhagic and stained the anterior corners of both eyes or even both eyelids, spectacle-like.   Melena and profuse hematuria were rather rare occurrences.   Mild hematuria is not necessarily connected with the hemorrhagic disease and can be observed as an independent complication, without any other hemorrhagic symptoms, in rats kept on a vitamin B_6 free basal diet.

The most impressive manifestations of the hemorrhagic diathesis in question were blood effusions into the skin, exhibiting all the characteristics of purpura (Fig. 1).   In the gross there were patches of purpura of varying size, some confluent, resulting in larger areas of dark red or blue discoloration.   These occurred mainly on the dorsum of the feet and, particularly, on the hind legs or on the face and neck.   The first but rather hidden purpuric patches appeared usually on the back over the scapula or over the ribs close to the spine.   We have observed pathologic disturbance of hematopoiesis in 72 rats thus far.   Of these animals, 46 displayed visible purpura of a more or less severe degree.   As a rule, purpura is a late manifestation of this specific disease.   When it appears it gradually increases in severity until the fatal issue which normally follows in from 1 to 2 days.

Blood Morphology

Examination of the blood[4] of rats in the acute stage of hemorrhagic diathesis revealed (Table I), in the majority of the animals, low percentage of hemoglobin

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[4] Blood was obtained by snipping off the tip of the tail after it had been soaked for a minute in warm water.   Hemoglobin percentage was determined by the Sabli hemoglobinometer.   Red and white blood cells were counted according to standard methods, using the improved Neubauer counting chamber.   Wright's stain was used for differential counts and brilliant cresyl blue for determination of reticulocyte percentage.

Platelets were counted according to the direct method involving the use of a hemacytometer.   Blood was drawn up in the red cell-counting pipet to the mark 0.5 and diluted with Rees and Ecker's (9) diluting fluid to the mark 101.   The platelets were then counted as in doing a red cell count and with the same calculations.

TABLE I

Blood Findings in Rats with Panmyelophthisis

Rat No.	Date	Hemo-globin	R.B.C. per c.mm.	W.B.C. per c.mm.	Platelets per c.mm.	Differential count				Nucleated red cells per 100 W.B.C.	Reticulocytes per 100 R.B.C.
						Polymorphonu-clears	Lymphocytes	Monocytes	Eosinophiles		
		per cent	*millions*			*per cent*	*per cent*	*per cent*	*per cent*		
6627	July 2	46	4.83	—	—	—	—	—	—	—	—
6798	" 6	20	—	1,900*	—	—	—	—	—	—	—
6873	Sept. 4	46	—	5,200	—	—	—	—	—	—	—
	" 5	33	3.17	11,200	230,000	—	—	—	—	—	—
7098	Nov. 28	40	4.95	600	20,000	0	100	0	0	—	—
7129	" 21	55	—	700	—	0	100	0	0	—	—
7130	" 29	35	—	1,100	—	0	96	4	0	—	—
7132	" 27	—	—	600	—	12	88	0	0	—	—
7183	" 29	—	—	—	—	8	80	12	0	—	—
7296	" 28	—	—	550	—	4	75	20	1	—	—
7297	" 28	31	—	1,100	—	4	96	0	0	—	0.1
7298	Dec. 1	52	6.06	750	—	2	96	2	0	—	7.0
	" 4	—	—	<500	—	0	100	0	0	900	—
7300	Nov. 27	55	6.70	1,300	130,000	12	88	0	0	—	—
	" 28	26	—	800	—	8	92	0	0	1	6.0
7344	Dec. 5	69	7.83	600	50,000	0	100	0	0	—	—
7438	Mar. 3	73	8.20	1,100	130,000	4	92	2	2	—	—
	" 5	—	7.99	670	150,000	2	94	2	2	16	—
7553	Nov. 30	—	—	—	—	0	96	4	0	36	—
7570	Jan. 13	55	6.06	900*	40,000	8	88	4	0	5	4
7574	Feb. 4	60	9.30	2,450	200,000	0	94	6	0	—	—
	" 5	—	—	—	—	0	80	20	0	—	—
7577	Nov. 30	30	3.03	920	70,000	4	88	0	8	568	—
7675	Apr. 11	65	8.07	1,300	80,000	0	80	20	0	10	—
7716	" 10	60	6.35	750	40,000	4	76	20	0	24	—
7759	" 7	88	9.50	3,500*	90,000	6	89	4	1	—	—
	" 8	78	7.38	2,300*	80,000	2	96	2	0	—	—
7760	Mar. 24	49	5.80	1,150*	60,000	2	80	18	0	6	—
7765	" 22	—	4.62	1,800*	40,000	0	82	18	0	4	—
7766	Apr. 3	—	—	1,200	—	1	86	13	0	1	—
7770	Mar. 24	46	4.75	1,300*	20,000	0	100	0	0	—	—
7783	Apr. 13	52	5.40	2,600	60,000	7	88	5	0	7	—
7809	" 11	—	—	—	—	4	86	10	0	22	—

*Uncorrected.

TABLE I—Concluded

Rat No.	Date	Hemo-globin	R.B.C. per c.mm.	W.B.C. per c.mm.	Platelets per c mm.	Polymorphonu-clears	Lymphocytes	Monocytes	Eosinophils	Nucleated red cells per 100 W.B.C.	Reticulocytes per 100 R.B.C.
		per cent	millions			per cent	per cent	per cent	per cent		
7853	Apr. 29	10	1.47	370	30,000	0	100	0	0	250	—
7867	" 26	83	9.10	330	100,000	0	89	11	0	4	—
7870	" 9	—	8.02	1,550	30,000	4	80	16	0	—	—
7884	" 16	48	—	1,000	140,000	0	92	8	0	32	—
7914	Mar. 30	31	—	1,800*		4	84	12	0	—	—
7948	Apr. 5	39	4.16	600	70,000	0	100	0	0	50	—
8033	May 21	83	8.10	1,900	20,000	0	86	14	0	5	—
8039	" 12	39	4.42	1,250	10,000	0	89	11	0	7	—
8040	" 23	54	5.45	850	70,000	0	76	24	0	12	—
8044	" 14	42	4.15	900	30,000	2	93	5	0	6	—
8105	" 15	—	4.95	1,150	90,000	0	84	16	0	4	—
8140	" 21	58	7.00	1,400	120,000	3	82	15	0	7	—
8152	" 25	54	4.90	1,250	50,000	0	92	8	0	30	—

and low platelet, white blood cell and red blood cell counts, with a very pronounced granulocytopenia. The leucopenia and the disappearance of the granulocytes may even he regarded as regular features of the disease and were missing in only 2 rats (6873 in Tahle I and 7237 in Table III) in the whole series. The polymorphonuclear cells when present were always segmented, frequently showing vacuolation and other signs of progressive disintegration.

In the control animals, which were kept on the same vitamin B₆ free diet as were the anemic rats, we found constantly high hemoglobin, red blood cell and white blood cell values, with a very distinct *granulocytosis* (Table II). These animals had no signs of a pathological impaired hematopoiesis, whether they did or did not display symptoms of acrodynia. In view of the generally very high platelet count in rats that had no hemorrhagic manifestations (Tables II and III), a count of 100,000 or less can be considered pathologically diminished. This assumption is borne out by the fact that the bleeding time after clipping the tail to obtain blood for examination, in rats with a platelet count of 100,000 or less, was very markedly prolonged. The clotting time was as a rule within normal limits.

From the foregoing summary of the hemorrhagic manifestations observed and the morphologic blood findings, it becomes evident that we are dealing here with a profound disturbance of the primary blood-

producing tissue, the reticulo-endothelium, in its transformation into the three distinct types of blood cells, *viz.*, into red blood cells, white blood cells and megakaryocytes (platelets). Generally the disease is ushered in by granulocytopenia but is followed soon by thrombocytopenia, affecting the red blood cells only at a later stage in the form of a progressive anemia. This combination of symptoms and their consecutive appearance are characteristic of the clinical entity called

TABLE II

Blood Findings in Control Rats

Rat No.	Date	Hemo-globin	R.B.C. per c.mm.	W.B.C. per c.mm.	Platelets per c.mm.	Polymorphonuclears	Lymphocytes	Monocytes	Eosinophiles	Nucleated red cells per 100 W.B.C.	Reticulocytes per 100 R.B.C.
		per cent	*millions*			*per cent*	*per cent*	*per cent*	*per cent*		
6331	Jan. 14	—	9.28	21,000	1,000,000	68	32	0	0	—	—
6547	Dec. 21	100	11.98	13,800	140,000	86	14	0	0	—	—
6611	Apr. 22	104	11.90	22,700	580,000	76	18	6	0	—	—
6770	Dec. 2	—	—	10,550	—	72	25.5	2.5	0	—	—
6909	" 7	98	9.95	12,700	540,000	53	43	4	0	—	—
6943	" 11	90	9.52	6,700	710,000	50	49	1	0	—	—
6957	Aug. 13	95	12.31	16,400	—	80	19	1	0	—	—
6959	Dec. 18	72	9.67	15,500	170,000	57	35	8	0	—	—
7126	" 4	90	9.10	12,900	440,000	37	60	2	1	1	—
7203	" 2	—	—	—	—	47	52	1	0	—	—
7348	Jan. 12	105	14.25	11,900	1,020,000	80	18	2	0	—	<0.1
7696	Mar. 5	82	9.93	10,400	830,000	54	45	1	0	2	—

by Frank (10) aleukia hemorrhagica, which again is more or less synonymous with the term aplastic anemia.

The hematological data given in Tables I and III suggest in 2 rats already mentioned (6873 in Table I and especially 7237 in Table III) the presence of pure red cell anemia as a sign of a lesion involving only the red cell-producing system. In exceptional cases, for instance in rats 7438, 7574 and 7867 (Table I), granulocytopenia seems to be the leading symptom, and platelet production and erythropoiesis are normally active or are interfered with only to a mild degree. This manifestation could be classified as uncomplicated agranulocytosis.

Anatomical and Histological Examination

Mucous Membranes.—Necrotic and ulcerative changes around the mouth (Fig. 2) and on the mucous membranes, almost specific signs of human agranulocytosis, were observed rather infrequently in the rats which displayed signs of granulocytopenia. Furthermore, changes of this kind have been encountered in rats that were kept on the vitamin B_6 free basal diet but did not show signs of disturbed hematopoiesis.

Bone Marrow.—Fresh biopsy specimens of bone marrow (femur, humerus, vertebrae) from a healthy animal showed that the marrow was very cellular and included cells of all three blood systems: white blood cells, red blood cells and megakaryocytes. In contrast to this normal finding, bone marrow smears from femur, humerus and vertebrae of a rat which succumbed to the experimental aleukia hemorrhagica (rat 7570) showed distinctly reduced cell content. In the gross the bone marrow cavities seemed to be filled with fluid blood. The bone marrow of the vertebrae and femur may be considered very hypoplastic, and that of the humerus even more so. Myelocytes and myeloblasts were only rarely seen in the smears. Megakaryocytes were missing in the humerus and vertebrae; only one was found in the femoral marrow. Normoblasts and early and late erythroblasts were present and were more common than cells of the granulocytic series. There were also phagocytic cells and, in the vertebral marrow, cells that were apparently lymphoid.

Similar findings were recorded in fresh bone marrow smears obtained from a second animal that was in a rather less advanced stage of the disease (rat 7870).

The results of the examination of fresh bone marrow have been substantiated by histological studies carried out on 25 rats in diverse stages of the disease. Appearance of the marrow varied in different bones and in the bones of different animals. In some, the only abnormalities were intense hyperemia, almost entire absence of megakaryocytes and a reduction in the number of cells, chiefly the granulocytes, in the marrow (Fig. 3). In others, in addition to the severe hyperemia, there was a varying amount of hemorrhage and edema, accompanied by more pronounced reduction in the number of cells in the marrow, and the granulocytes and megakaryocytes were altogether missing. Many of the patches of edema contained deposits of fibrin (Fig. 4). Small deposits of brown pigment, which did not give the reaction for iron but in many instances showed crystalline formation in the characteristic burrs of hematoidin, were repeatedly found. In the marrow of some bones of most of the animals and in that of all the long bones of some of the animals megakaryocytes and granulocytes were entirely absent and the hematopoietic cells in the tissue of the marrow had almost completely disappeared (Fig. 5).

Suprarenal Bodies.—A frequent postmortem finding in the internal organs, and the most striking, was hyperemia and hemorrhage in the suprarenal bodies (Fig. 6). The hemorrhage was easily recognizable in 24 out of 72 animals and

varied in extent from small patches, in the cortex or medulla or both, to diffuse hemorrhage with obliteration of the natural architecture of the entire organ.

Microscopically, in the regions of hyperemia alone, the vessels were distended with blood and the cells between them showed a varying degree and amount of degenerative change. In those parts where the hyperemia was intense and also in the regions of extravasation of blood, the parenchyma was almost or entirely destroyed. The destruction of parenchyma was evidently secondary to the hyperemia or hemorrhage because in the parts that were not affected by these processes the parenchyma was quite well preserved (Fig. 7).

In the hyperemic and hemorrhagic suprarenal bodies of 2 of the animals there were masses of bacteria in the cortex but no surrounding inflammation. Most of the masses of bacteria were within the blood vessels, and the parenchyma immediately surrounding them showed coagulation necrosis.

Kidneys.—Only 2 of the animals showed abnormalities recognizable in the gross. These consisted of massive necrosis of the upper pole of both kidneys in one animal and patches of necrosis in the upper pole of one kidney in the other animal. They were the same animals in which the suprarenal bodies also showed foci of necrosis with masses of bacteria in them. The kidneys of some of the animals showed a varying amount of hyperemia and some were slightly swollen, but many appeared quite normal.

Microscopically, the only change of a general nature was a varying degree of degeneration of the tubular epithelium, which in no case was very severe. In the portion of the kidneys of the 2 animals necrotic in the gross, the tissue showed the typical picture of coagulation necrosis. In this necrotic tissue, however, and mostly within the dilated blood vessels there were masses of bacteria. Surrounding these bacterial masses there was no inflammatory reaction. With the exception of the bacterial emboli no other cause of the necrosis was observed in the sections. The large blood vessels were patent.

Spleen.—At macroscopical examination the spleen showed only hyperemia. There were no striking changes of size and no other characteristics recognizable in the gross.

Microscopically the sinusoids were distended with blood. In the pulp, especially around the lymphoid follicles (Fig. 8), there were zones of hemorrhage. Most of the follicles had no germinal centers. The most striking abnormality was the absence of megakaryocytes. In the pulp there was moderate cellularity with a varying amount of histiocytic hyperplasia and fibrosis (Fig. 9). In the zones of hemorrhage the pulp cells showed varying degrees of degenerative change but in other portions they were well preserved. In the pulp of some of the spleens there were small deposits of brown pigment similar to those in the bone marrow.

Intestine.—In several animals that had clinical melena the large intestine showed, microscopically, some extravasation of blood in the mucosa without accompanying inflammation.

Testes.—In 2 of the rats the testes, in the gross, were intensely hyperemic. Microscopically, these testes showed great dilatation of the blood vessels and

some extravasation of blood in the interstitial tissue. The parenchyma was well preserved, but there were no spermatozoa in the lumen of the tubules.

Skin.—Microscopically, in the regions of purpura, the epidermis varied in thickness. Especially was this true of the stratum granulosum and the stratum corneum. The stratum spinosum showed varying degrees of parakeratosis and ̶̶ ̶̶ presumably as the last remnant of the acrodynia-like skin manifesta- ̶̶ Extravasation of blood ̶̶ ̶̶ ̶̶ There tion ̶̶ rium and was no deposition of blood pig̶̶ ̶̶ ̶̶ The deeper por̶̶ ̶̶ apparent around the extravasated blood. The deeper por̶̶ the subcutaneous tissue showed only occasional small foci of hemorrhage. The upper part of the corium was hyperemic and edematous (Fig. 10).

The anatomical and histological findings are in complete accordance with the conclusions drawn from observations made on the sick animals and from the appearance of the blood smears. In brief, it becomes evident that this experimental disease in the rat, having for its anatomical basis a more or less advanced panmyelophthisis, resembles most closely the characteristic syndrome of aleukia hemorrhagica in man. As to its cause, three possibilities have to be taken into consideration: (a) nutritional etiology, (b) bacteriological etiology or (c) eventually a combination of both,—bacterial infection with an underlying nutritional factor.

Bacteriological Studies

Bacteriological studies[5] have been carried out to clarify the question of a possible infection. 5 rats were examined, all of which were in a rather advanced stage of the disease. Blood, obtained by heart puncture, and spleen tissue served as material; brain broth, blood agar plates and Endo plates were used as culture media. The results were not conclusive and were even rather negative. In 2 rats slightly pathogenic *Staphylococcus aureus* was found; in one rat the bacteriological culture yielded apathogenic staphylococcus, in one rat *Bacillus coli* and *Bacillus proteus*, and in the 5th rat the bacteriological cultures were completely negative. Anemia and hemorrhagic manifestations were not observed when intraperitoneal injection was made into 2 rats of 0.3 cc. of blood, taken again by heart puncture from a moribund anemic animal (rat 7159), or when injection was made into eight rats of 0.5 cc. of a 48 hour brain broth culture of mannite-fermenting *Staphylococcus aureus* obtained from a rat that showed typical panmyelophthisis (rat 7298).

The 2 rats that received the blood of rat 7159 and 6 of the 8 rats injected with the brain broth culture of the staphylococcus isolated from rat 7298 were on the same vitamin B_6 free diet that was fed the experimental animals. Several displayed specific symptoms of acrodynia. The basal diet of the 2 remaining injected rats was deficient in lactoflavin; that is, it was supplemented with vitamin B_1 and Peters' eluate but not with lactoflavin. One rat on the vitamin B_6 free diet died in the first 12 hours after intraperitoneal injection of the brain broth culture without exhibiting any sign of anemia or hemorrhagic diathesis. The remaining 7 animals on the B_6 deficient diet and the 2 on the lactoflavin deficient diet were observed for several weeks. No untoward effect was seen except for a slight local abscess formation at the place of injection of the brain broth culture in some of the rats, accompanied by temporary loss in weight. Blood examination 24 hours after injection revealed no alteration in the direction of panmyelophthisis.

	Rat 6331 (B₆ deficient diet)	Rat 7038 (Lactoflavin deficient diet)	Rat 7076 (Lactoflavin deficient diet)
Hemoglobin, *per cent*.....................	94	100	88
Red blood cells, per c.mm., *millions*.........	10.6	8.8	8.0
White blood cells, per c. mm...............	23,900	12,400	8,800
Differential count:			
Polymorphonuclears, *per cent*...........	83	63	35
Lymphocytes, *per cent*..................	14	36	65
Monocytes, *per cent*......	3	1	—
Platelets, per c. mm......................	310,000	290,000	150,000

In view of these facts we are inclined to believe that the bacterial invasion found in the majority of the panmyelophthisic rats examined for bacteria was a secondary process due to generally lowered tissue resistance. The same conclusion may be drawn with regard to the coagulation necrosis in the suprarenal glands and in the kidneys in which bacteriological emboli were found during histological examination, as mentioned above. The absence of more pronounced inflammatory changes is only a natural consequence of the breakdown in cellular defense and tallies well with the histological picture in human agranulocytosis given by Piette (11).

Great care was taken to exclude the possibility of *Bartonella* infection, which has been proved to be an important and frequently even a misleading complication in rats (12) and in dogs (13). The anemia produced by *Bartonella* infection in rats and dogs is always accompanied by leucocytosis, in particular by granulocytosis. Hemorrhagic manifestations were never recorded. In our anemic rats that manifested hemorrhagic diathesis and granulocytopenia, *Bartonella* bodies were missing, as was to be expected.

The negative or at least inconclusive results of the bacteriological search for the possible etiology of the panmyelophthisis encountered

in our rats bring again to the foreground the possibility that a nutritional deficiency is the main causative factor.

Summary of Experimental Data

In previous studies on the vitamin B_2 complex reported by one of us (3) in which a synthetic diet was used consisting of rice starch, casein (Glaxo), melted butter fat, cod liver oil, salt mixture and, as supplements, purified vitamin B_1 and lactoflavin preparations, the syndrome of anemia and hemorrhagic diathesis was never observed. In the studies now reported, rice starch was replaced in the diet by cane sugar and the purified lactoflavin preparation by crystalline natural, later synthetic, lactoflavin, while the vitamin B_1 concentrate was of the same source and of the same degree of purification as that used in our previous experiments. Under these conditions panmyelophthisis was noticed in 33 animals. Of the rats which manifested acrodynia when they were kept on the vitamin B_6 free basal diet and which were subsequently treated with purified vitamin B_6 concentrate prepared[6] from wheat germ, cane molasses and rice polishing, 39 developed anemia and hemorrhages. In contrast to these facts, signs of aregenerative anemia were completely missing in vitamin B_6 deficient rats that died from acrodynia or in those that were treated with cruder B_6 concentrates such as Peters' eluate, yeast, cow's milk, human milk (both milks in daily doses ≥ 3.0 cc.), liver or wheat germ autolysate. Anemia and hemorrhagic manifestations were also absent in rats that received, in addition to the vitamin B_6 free diet plus vitamin B_1 concentrate, Peters' eluate instead of lactoflavin. The lactoflavin deficient rats remained under observation for several months and never showed any signs of pathologic hematopoiesis or hemorrhages. This group included 85 animals. Of these, 49 have been successfully treated with lactoflavin or with human milk and cow's milk, 16

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[6] These B_6 preparations represented different degrees of concentration. In the case of wheat germ the purification was extended generally over autolysis, precipitation with lead acetate, adsorption on fuller's earth, elution with barium hydroxide to precipitation with phosphotungstic acid and regeneration of this precipitate with barium hydroxide. Details are given in the paper by Birch and György (7). Similar preparations have been made from cane molasses and rice polishing.

succumbed to the lactoflavin deficiency, 6 to diarrhea, 2 to pneumonia and 12 to other infections.

As to the incidence of panmyelophthisis, it has to be borne in mind that the current experiments served primarily for purification of vitamin B_6. The group of vitamin B_6 deficient rats included 319 animals. Of these 52 were cured of acrodynia, 70 died from the same disease, 41 succumbed to pneumonia, 30 to septic infections comprising kidney and lung abscesses, 54 to diarrhea and other infections the cause of which could not be determined and, finally, 72 developed panmyelophthisis. This number appears to be a high percentage considering the fact that in the majority of animals the B_6 deficiency was not eliminated and acted as a complicating factor.

Panmyelophthisis developed in several of our animals that had been previously treated with vitamin B_6 preparations of varying purity and cured of the B_6 deficiency disease. They were kept continuously on the basal diet after treatment had been discontinued. In view of these complications it is difficult to evaluate the significance of the length of the preparatory period needed for the development of the hemorrhagic disease in question. We have observed the onset of the disease after the following periods of time had elapsed.

Number of weeks elapsed..............	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	26	28
Number of rats in which disease onset was observed.........	1	1	3	3	5	6	6	7	8	9	3	1	4	2	3	2	4	2	1	1

Thus, on an average, 14 weeks elapsed before the first symptoms of panmyelophthisis could be noticed, although they might occur after 5 to 6 weeks of a preparatory period.

As to the influence of sex, panmyelophthisis was observed in almost even distribution: 38 female and 34 male animals.

Seasonal variations were a more pronounced factor. There were two distinct peaks, one in spring and one in late fall, with a definite minimum in August, September and October. The period of observations covered 12 months, from June, 1936, through May, 1937.

June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May
2	5	0	2	0	15	6	1	4	5	12	. 20

The influence of seasonal variations cannot be explained by temperature differences, for the temperature of the animal room was kept at a fairly constant level, except perhaps on days of very warm outside temperature when regulation was not possible. In view of the depressing influence of cold environmental temperature on the bone marrow, shown by Huggins and Blocksom (14), one would have suspected that the same physical factor should at least accelerate the production of panmyelophthisis. Contrary to these expectations, out of 34 rats which for varying periods up to 26 weeks we exposed daily between 5 p.m. and 9 a.m. to cold temperature (about 40°F.) only one animal developed anemia (rat 7237 in Table III). Surprisingly, the same rat was one of the 2 animals that manifested "pure red cell anemia" without diminution of the white cell and platelet counts.[7]

The fact that panmyelophthisis has never occurred in rats that have received Peters' eluate was considered by us a possible clue to the nutritional etiology of panmyelophthisis. In order to prove this assumption we tried administering Peters' eluate to several rats ill with panmyelophthisis. Unfortunately many animals were already in a moribund state when the very acute, usually almost fulminant breakdown in hematopoiesis was discovered. They died despite treatment, from 1 to 2 days after it had been initiated, frequently refusing any medication or food. In animals in which aleukia hemorrhagica was diagnosed at an early stage, administration of Peters' eluate in doses corresponding to 10 to 100 gm. of fresh yeast daily prevented the fatal issue and restored normal conditions. Out of 12 rats which survived the first 2 days of treatment, 4 died with very severe manifestations of progressing aleukia hemorrhagica (3 on the 3rd and 1 on the 4th day of the therapeutic assay). 8, however, were saved. They showed a gradual improvement in the morphologic blood composition (see Table III) and in the permeability of the vessels (purpura). It is of interest to note that in rat 7882 the increase in the white cell count and in the granulocyte count was the first sign that a positive effect was exercised by Peters' eluate, the hemoglobin value, red cell count and platelet count at the same time having still shown a decline. In rats 7811 and 7984 subcutaneous medication proved equally beneficial.

[7] In contrast to the negative results with regard to the possibility of accelerated production of panmyelophthisis, symptoms of acrodynia appeared distinctly earlier in the cold-treated rats than in the control animals (15).

In comparison to the pathologic findings in the animals ill with panmyelophthisis, it is of essential importance to note that in rat 6946, which had made a complete recovery after treatment, the microscopic appearance of the bone marrow and of

TABLE III

Blood Findings before and after Specific Treatment of Panmyelophthisis in Rats

Values recorded after treatment are in italics

Rat No.	Date	Hemo-globin	R.B.C. per c.mm.	W.B.C. per c.mm.	Platelets per c.mm.	Differential count				Nucleated red cells per 100 W.B.C.	Treatment (Peters' eluate)
						Polymorphonu-clears	Lymphocytes	Monocytes	Eosinophiles		
		per cent	*mil-lions*			*per cent*	*per cent*	*per cent*	*per cent*		
6946	Dec. 4	72	7.53	2,600	16,000	6	93	1	—	—	
	" *15*	*68*	*6.17*	*8,400*	*1,010,000*	*48*	*51*	*1*	—	—	By mouth
7237	Feb. 3	41	3.80	11,400	440,000	61	39	—	—	8	
	" 6	30	2.97	4,650*	310,000	28	53	18	1	Numer-ous	
	" *9*	*43*	*3.60*	*5,100*	*280,000*	*31*	*64*	*4*	—	—	By mouth
	" *13*	*76*	*5.43*	*4,400*	*440,000*	*40*	*55*	*4*	*1*	*4*	
7703	" 6	45	4.12	1,950	60,000	4	76	20	—	—	
	" *9*	*38*	—	*2,100*	—	*32*	*48*	*20*	—	—	By mouth
	" *13*	*60*	*5.28*	*5,350**	*340,000*	*71*	*25*	*4*	—	*12*	
7579	Mar. 25	42	4.05	1,800	60,000	8	80	11	1	80	
	Apr. 1	*72*	*5.10*	*11,000*	*210,000*	*59*	*33*	*8*	—	*3*	By mouth
7811	May 2	—	—	1,100	—	3	81	16	—	12	
	" *10*	—	—	*11,400*	—	*49*	*43*	*7*	*1*	—	By injection
	" *18*	*81*	*6.13*	*12,200*	*610,000*	*50*	*44*	*5*	*1*	—	
7882	" 1	73	10.40	1,975	110,000	2	87	11	—	12	
	" *6*	*57*	*5.58*	*5,965*	*20,000*	*11*	*81*	*4*	*4*	*21*	By mouth
	" *18*	*84*	*6.18*	*8,600*	*1,000,000*	*21*	*73*	*5*	*1*	*2*	
7984	" 15	57	5.85	600	70,000	2	76	22	—	11	
	" *24*	*65*	*4.68*	*3,500*	*180,000*	*55*	*34*	*7*	*4*	*1*	By injection

*Uncorrected.

the spleen was entirely normal (Figs. 11 and 12), with numerous megakaryocytes and other blood cell-producing elements.[8]

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[8] An incidental finding in the autopsy specimens of bone marrow and spleen from one patient who received Peters' eluate was a great increase in the number of megakaryocytes and megakaryoblasts. We do not attempt to evaluate this finding, but it is of interest because of similar findings in rats treated with the same preparation.

DISCUSSION

From the foregoing résumé of the experimental findings recorded for 72 rats it becomes evident that a new disease of the rat has been found. The hematological data, controlled by study of fresh bone marrow smears and by histological examination of the bone marrow and the spleen, together with a great variety of hemorrhagic manifestations, correspond to the classical syndrome of aplastic (aregenerative) anemia (aleukia bemorrhagica). It is now generally accepted that the human counterpart of this disease, aplastic anemia, or panmyelophthisis as its histological equivalent, is the final combined result of three more or less separate, pathologic reactions involving transformation of reticulo-endothelial cells into (a) red blood cells, (b) white blood cells and (c) megakaryocytes. This conception is borne out by the fact that morbid conditions that affect only one specific system of blood cell production are widely known in human pathology; we have (a) agranulocytosis or granulocytopenia from deficient production of white cells, especially of granulocytes, (b) thrombocytopenia from lack of platelets and (c) pure red cell anemia from diminished production of erythrocytes caused by arrest in maturation of red blood cells in the first stage of their formation.

These considerations apply equally well to our animal experiments, especially with regard to the gradual transition of a partial aplasia, as, for instance, agranulocytosis into fully developed panmyelophthisis.

In rats, partial or total arrest in maturation of the reticulo-endothelial cells has been produced by nutritional deficiency, and in particular by lack of one constituent of the vitamin B_2 complex, a component which in this connection may be regarded as a specific maturation factor. In some animals panmyelophthisis developed when they were kept on the diet used by us, usually after spontaneous improvement of a mild acrodynia which had just started. In the greater part of our observations, however, the specific blood dyscrasia followed the addition of a more or less purified vitamin B_6 concentrate, which was administered in order to cure the acrodynia in progress. In the majority of these cases the vitamin B_6 deficiency disease (acrodynia) was in distinct remission, or even completely cured, before symptoms of aleukia hemorrhagica became manifest. Therefore we have to infer that the maturation factor must be different from vitamin B_6.

Furthermore, in uncomplicated deficiency of the filtrate factor, symptoms of anemia or of a hemorrhagic diathesis were not noticed and not reported (5). We could neither prevent nor cure panmyelophthisis merely by addition of the fuller's earth filtrate of a rice bran extract which was supposed to be rich in the filtrate factor (5). We believe, therefore, that this factor cannot be the determining cause of the specific disturbance in hematopoiesis. In our opinion it appears more likely that the maturation arrest in the hematopoietic tissues that occurred as a preliminary reaction to panmyelophthisis in our rats is related to lack of a hitherto unknown factor (or factors) of the vitamin B_2 complex.

Such a theory of specific nutritional disturbance is borne out by the fact that panmyelophthisis was regularly prevented and, in cases where it was recognized in an early stage, also cured by a watery yeast extract concentrate, represented by Peters' eluate. It was not prevented or cured, however, by lactoflavin or by purified vitamin B_6 concentrates or by a supposedly active filtrate factor preparation.

It is a common occurrence in vitamin studies that only one disease becomes apparent in cases of a combined deficiency while the other deficiencies remain more or less suppressed. For instance, rats kept on a diet deficient in the whole vitamin B complex do not show symptoms of acrodynia but show merely symptoms of polyneuritis or even only of dystrophy. Production of acrodynia is accelerated by the addition of a sufficiently large amount of vitamin B_1 to the diet (Kellogg and Eddy (16), György (3)). By assuming the presence of a combined deficiency in the basal diet used in our present studies, we may surmise that, under the conditions chosen, vitamin B_6 deficiency was the dominant disease, while panmyelophthisis was relegated to a more latent position. This prevalence is not absolute, however, as we were able to observe in several animals in which the concurrence of the two diseases ended in favor of panmyelophthisis. The dominance of the vitamin B_6 deficiency also satisfactorily explains the fact that aplastic anemia was more frequently encountered in rats in which treatment of acrodynia had been instituted with purified B_6 concentrates (presumably deficient in the maturation factor or factors) than in the untreated rats.

Our investigations failed to find support for the assumption that, in the production of panmyelophthisis, specific infection in addition to basic nutritional disturbance plays an important etiologic rôle. Although this negative result does not exclude with final certainty the interaction of an undetermined bacteriological cause, which is then

subject to nutritional influence, we have to regard an interaction of this kind as a very remote possibility.

Whereas partial symptoms of panmyelophthisis, produced in animals by nutritional means, have been described prior to our own observations, they were either not recognized as such or at least were not recognized in their relation to the common denominator of maturation arrest in the primary blood-producing tissue, the reticulo-endothelium.

In chronological order the first contribution we have to mention is a paper by Shipley, McCollum and Simmonds (17) in which it is stated that in rats kept on a vitamin B complex free diet there might develop lesions in the bones "which are essentially identical with those seen in guinea pigs suffering from acute and uncomplicated scurvy," hemorrhages in the medullary cavity and, finally, complete destruction of the cellular marrow elements. Disappearance of the bone marrow generally precedes the hemorrhages. Hemorrhages in other organs and, in particular, purpura-like blood effusions were not described, however, and Happ (18), who carried out blood examinations on 2 rats of this group, failed to demonstrate even a slight degree of anemia but found some leucopenia, significantly without agranulocytosis. Shipley, McCollum and Simmonds attributed the pathologic findings in bone and bone marrow to beriberi and compared them with scorbutic manifestations in guinea pigs deficient in vitamin C. At the time of publication of their findings, the composite nature of vitamin B was not realized. In view of our recent observations we may consider that the bone marrow changes, including the medullary hemorrhages, were a *forme fruste* of panmyelophthisis and therefore were manifestations of deficiency of a specific maturation factor.

In nursing young of mother rats which during pregnancy and lactation were kept on a diet with an apparently suboptimal content of the vitamin B complex, present as wheat germ or yeast, Sure and Schilling (19) and Moore, Brodie and Hope (20) have reported, in addition to paralytic conditions, hemorrhages in the osteogenetic tissues and also, with far less regularity, in the subcutis (petechiae) and in the internal organs. Blood findings and histological bone marrow examinations were not reported.

More closely resembling our own observations, but still far from the fully developed picture of panmyelophthisis, are those recently described by Miller and Rhoads (21) in dogs and by Day, Langston and Shukers (22) in monkeys. The former authors emphasized the analogy of the clinical syndrome of agranulocytosis with the condition encountered in dogs fed a modification of the black tongue producing diet of Goldberger. Hematological data are given for 10 dogs. In only one dog, however, did the granulocytes drop to a level as low as 15 per cent. They were on the average >50 per cent, with a very definite total leucopenia. The histological picture of the bone marrow given by Miller and Rhoads showed also far from complete destruction of the granulocytes or other blood cell-producing elements.

In 6 young macaques, Day, Langston and Shukers obtained evidence of anemia and leucopenia resulting, in their opinion, from vitamin deficiency. Addition of yeast to the diet prevented the disturbance in morphologic blood composition. In general, as leucopenia developed in monkeys kept on the basal diet, "the decrease in white cells appeared to be more at the expense of the neutrophils than of the lymphocytes." But inasmuch as the differential counts were very variable, the authors admitted that "it is difficult to make any broad statement that holds true for all the animals." This statement excludes also the diagnosis of a true agranulocytosis, in spite of the unquestionable tendency to it.

Hemorrhages and low platelet counts, indispensable attributes of panmyelophthisis, were reported neither by Miller and Rhoads nor by Day, Langston and Shukers. In a personal communication Day points out that the hitherto unpublished platelet counts of his animals were usually within or only slightly below the physiological range during the disease but that they always dropped distinctly before death. Hemorrhages were absent even in this stage.

In spite of the incomplete symptomatology, the disturbance in hematopoiesis described by Day, Langston and Shukers in monkeys and by Miller and Rhoads in dogs may be considered to be probably related to the classical picture of aleukia hemorrhagica encountered by us in rats. This analogy is substantiated by the seemingly identical nutritional etiology.

Several authors (Witts (23), Beck (24), Fitz-Hugh (25)) have assumed that a hypothetical maturation factor influences the regulation of the primary blood production also in man. As to the origin of this factor, endogenous (hormonal) or exogenous (dietary) sources have been taken into consideration. For purely theoretical reasons Witts regarded the second possibility as more consistent with clinical facts, thus putting the pathogenesis of panmyelophthisis on a qualitative basis similar to that of pernicious anemia, although he was at the same time unable to identify the "maturation factor" for which he searched.[9]

However, in man this supposedly nutritional etiology represents, certainly to a greater degree than can be inferred from our animal experiments, at least in the majority of cases, merely the background or basis for the bone marrow depression and its clinical manifestations. Although it is now generally accepted that bacterial influence is of only secondary importance and that infections follow rather than precede aplastic anemia (Frank (10)) or agranulocytosis (Roberts and Kracke (26), Baldridge and Needles (27), Thums (28)), toxic factors

[9] See Witts's scheme of anhematopoietic anemias showing point of action of the substances essential for blood formation (23, page 549).

in many cases seem to activate or to release the chain of reactions leading finally to the clinical syndrome of aplastic anemia and its subgroups. Lately the presence of such a causal toxic influence, as well as that of the well known roentgenologic effect, has been proved for organic arsenic compounds (29), for benzene (30), dinitrophenol (31), gold preparations (32), quinine (33) and for amidopyrine (34), the latter particularly with regard to agranulocytosis.

The question whether these substances exert their toxic influence directly or through a secondary reaction, based on specific sensitivity, is not yet finally settled. The observations of Squier and Madison (35) as well as those of Disselmeyer and Zorn (36) seem to substantiate the presence of amidopyrine sensitivity in cases of agranulocytosis during or after medication with this drug. It is particularly interesting that administration of even one small dose of amidopyrine to these patients after they had recovered had a definite depressive action on the bone and on the bone marrow (35, 36, 37), an effect that could be closely followed in bone marrow smears of biopsy material (Plum (38)). The same deduction applies with regard to thrombocytopenic purpura that occurs as result of quinine sensitivity (33 b). On the other hand several authors, in the first place Kracke and his coworkers (34), maintain that most of the chemical compounds prominent in the etiology of panmyelophthisis and its subgroups act chiefly through their benzene ring which, after it is oxidized in the body, acquires direct toxic properties.

On the whole, the experimental production of agranulocytosis in animals was not successful in spite of several attempts. Infections (39), bacterial toxins (40) and also, with rare exceptions (41), the drugs (42) that played a definite rôle in suppression of the bone marrow function in man failed to produce conclusively relevant disturbances in animals.

We tried to produce panmyelophthisis by administering amidopyrine to rats kept under experimental conditions that were the same as those found necessary for spontaneous manifestation of the disease. Unfortunately we encountered difficulties in administration of amidopyrine to rats by mouth. It was impossible to pursue medication with 2 cc. of a 1 per cent solution of amidopyrine in 4 rats for longer than 1 week. To 2 other animals 0.5 cc. of the same solution was fed for 4 weeks. In all these 6 rats the results were completely negative. The morphologic blood picture presented normal data, and no symptoms of hemorrhagic diathesis could be detected. These negative results may be explained either by refractory behavior of our rats with regard to a directly toxic action of amidopyrine or by the fact that it is impossible to sensitize rats against this drug. The latter explanation

appears especially likely to us in view of the general unsuitableness of the rat for allergic experiments. But be that as it may, so far we have not been able to demonstrate in rats ailing with panmyelophthisis the presence of etiologically important toxic factors. Such non-toxic forms of the disease occur also not infrequently in man. The conclusion drawn as to the intimate relation between inhibited formation of bone marrow cells in man and that experimentally produced in rats is therefore valid for the time being for these non-toxic, so called idiopathic forms.

The experiments here reported cast new light on the so called Waterhouse-Friderichsen (W.-F.) syndrome that is characterized clinically by sudden onset, prostration, high fever, hyperpnea, rapidly fatal course and, as a special feature, by purpura in addition to monolateral or more frequently bilateral suprarenal hemorrhage found at postmortem examination. The first case was mentioned rather casually by Marchand (43) in 1880; later, Little (44), Dudgeon (45), Langmead (46) and others have given similar reports, but only since the analysis offered by Waterhouse (47) and later by Friderichsen (48) has its character as a specific and independent entity been recognized. The syndrome is usually encountered in young infants, from 6 months to 3 years of age, but it has also been found in older children and in adults (49). It is certainly different in its genesis from the suprarenal hemorrhage of the newly born, as the latter condition is determined essentially by purely mechanical causes (50).

Purpura, suprarenal hemorrhage, prostration and a rapidly fatal course frequently occurred in our rats as peculiarly grouped manifestations of panmyelophthisis. Furthermore, in a case of the W.-F. syndrome Glanzmann (51) found progressive thrombocytopenia and distinct diminution of the granulocytes with degenerative changes in the remaining polymorphonuclear cells. Although these blood findings seem to be rather exceptional, and leucocytosis prevailed in similar cases (50 b), the close analogy between the W.-F. syndrome and the appearance of some of our rats that were ill with panmyelophthisis can be regarded nevertheless as exceedingly striking.

Etiology of the W.-F. syndrome is now generally attributed to a septic infection and in particular, certainly in the majority of cases, to a fulminant meningococcus sepsis which ends fatally before the

local alterations of meningitis may develop. However, in rats ill
with the corresponding syndrome we were unable to prove the exist-
ence of a primary infection as a decisive etiologic factor. Whereas a
negative observation such as this does not exclude with certainty the
presence of an unrecognized infection, the nutritional control of the
production of the syndrome in rats permits us to make at least another
important suggestion with regard to its human equivalent. From the
rat experiments we may hypothesize that the bacterial, septic etiology
of the W.-F. syndrome in man is built up on the basis of a nutritional
deficiency similar to that which we have found necessary for the pro-
duction of the analogous disturbance in rats. With this assumption
it becomes conceivable why meningococci provoke the specific W.-F.
syndrome only in certain persons.

The possible identity of the maturation factor active in our rat
experiments with vitamin K (52) is ruled out by the morphologic
blood composition and the normal clotting time in rats ill with pan-
myelophthisis as well as by the difference in solubility, vitamin K
being fat-soluble, the maturation factor apparently water-soluble.
The same conclusion applies with regard to the identity of the matura-
tion factor with so called vitamin P (53), the biological function and
clinical use of which certainly seem to be different from those of our
maturation factor.

SUMMARY

During the 12 months ending May, 1937, 72 rats were observed
that manifested typical symptoms of panmyelophthisis. The disease
may start as agranulocytosis, thrombocytopenia or pure red cell
anemia, leading progressively, often rapidly, to aleukia hemorrhagica
with its typical manifestations (epistaxis, melena, hematuria, purpura).

Blood examinations revealed correspondingly low white cell, red
cell and platelet counts with very pronounced granulocytopenia (0
to 4 per cent). Bone marrow smears and histological findings were
consistent with the diagnosis of panmyelophthisis.

Suprarenal hemorrhage was a frequent postmortem finding.

The pathogenesis of this experimental panmyelophthisis and this
hemorrhagic diathesis is confined to special nutritional conditions.
These diseases have been observed by us in rats kept on a diet deficient

in vitamin B_6, containing cane sugar and supplemented with vitamin B_1 and crystalline natural or synthetic lactoflavin. Lack of vitamin B_6, however, is not a necessary condition, since the disease was encountered in the majority of the animals after the specific deficiency disease which became apparent in rats kept on the B_6 free diet was successfully treated with purified B_6 preparations. Even in the untreated animals kept on the B_6 deficient diet acrodynia was, as a rule, in distinct remission before symptoms of panmyelophthisis and hemorrhagic diathesis became manifest.

By means of the addition of Peters' eluate to the basal diet, panmyelophthisis could be prevented and, in animals where it was recognized in an early stage, cured. In view of these facts it is suggested that Peters' eluate contains a specific maturation factor for the primary blood-producing tissue, the reticulo-endothelium, a factor which, being different from lactoflavin, vitamin B_6 and probably also from the so called filtrate factor, constitutes another distinct component of the vitamin B_2 group.

Bacteriological studies brought forward no conclusive positive evidence for the infectious etiology of the experimental panmyelophthisis in our rats.

The possible relation of this new disease in rats to aleukia hemorrhagica and its partial manifestations in man, as well as to the so called Waterhouse-Friderichsen syndrome, is discussed.

Administration of amidopyrine, at least under the conditions chosen, failed to provoke panmyelophthisis in rats kept on the same diet as that given to rats in which the disease spontaneously developed.

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EXPLANATION OF PLATES

PLATE 15

FIG. 1. Hemorrhagic diathesis in rat 6722, showing effusion of blood into the skin (typical purpura).

FIG. 2. Noma-like gangrene in and around the mouth of rat 7703, seen in advanced stage of panmyelophthisis.
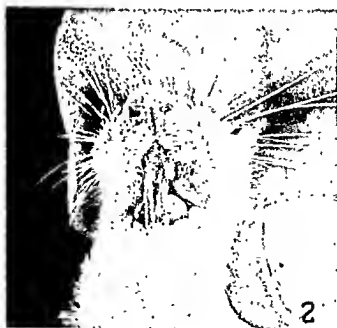
FIG. 3. Section of bone marrow from humerus of rat 7152; Giemsa stain used. There is intense hyperemia, with moderate edema. Marrow cells are greatly decreased in number and megakaryocytes or granulocytes are almost entirely absent. × 297.

FIG. 4. Section of bone marrow from humerus of rat 7133; Giemsa stain used. Varying amount of hemorrhage and edema is shown, with deposits of fibrin in many of the patches of edema. × 297.

FIG. 5. Section of bone marrow from humerus of rat 7130, stained with hematoxylin and eosin, showing exhaustion of marrow and severe edema. There is some deposition of fibrin. Marrow cells have almost completely disappeared, and no megakaryocytes are seen. There is some extravasation of red blood corpuscles. × 297.
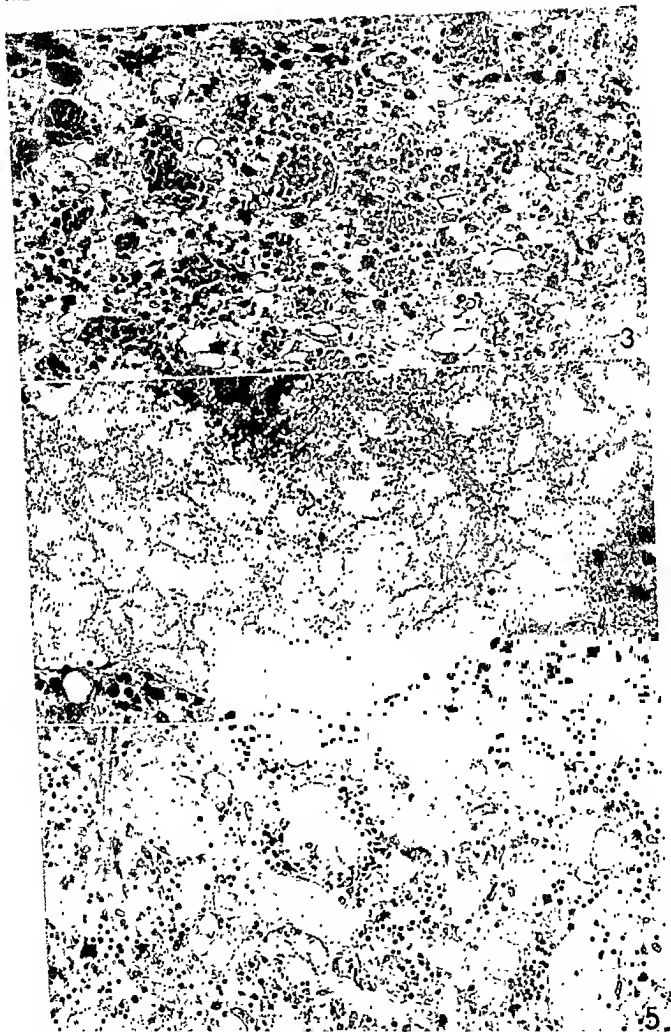
FIG. 6. Entire section of suprarenal body from rat 6722, stained with hematoxylin and eosin, showing patchy hemorrage and diffuse hyperemia. ×60.

FIG. 7. Section of cortex of suprarenal body shown in Fig. 6. Degeneration of cortical cells is seen in the region of hemorrhage and intense hyperemia. × 199.

FIG. 8. Section of spleen from rat 6227; Giemsa stain used. Sinusoids are distended with blood and zones of hemorrhage are seen around the lymphoid follicles. Megakaryocytes are absent. × 158.

FIG. 9. Section of spleen from rat 6383, stained with hematoxylin and eosin, showing moderate cellularity in the pulp, with varying amount of hyperplasia of histiocytes. × 280.
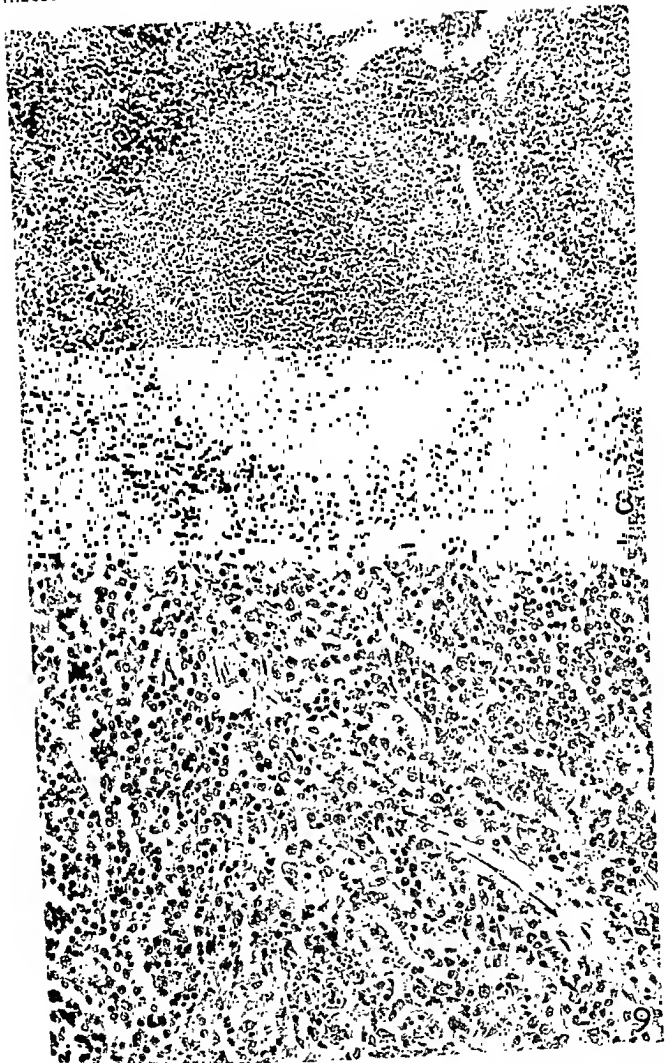
FIG. 10. Section of skin of rat 7159, stained with hematoxylin and eosin, showing hyperemia, hemorrhage and edema in upper portion of corium.   × 100.

PLATE 19

PLATE 20

Fig. 11. Section of bone marrow from humerus of rat 6946 which had made a complete recovery after treatment. Hematoxylin and eosin stain used. There is a normal number of megakaryocytes and the cellular marrow shows many granulocytes. × 570.

Fig. 12. Section of spleen from rat 6946 which had completely recovered after treatment. There is a normal number of megakaryocytes. × 495.
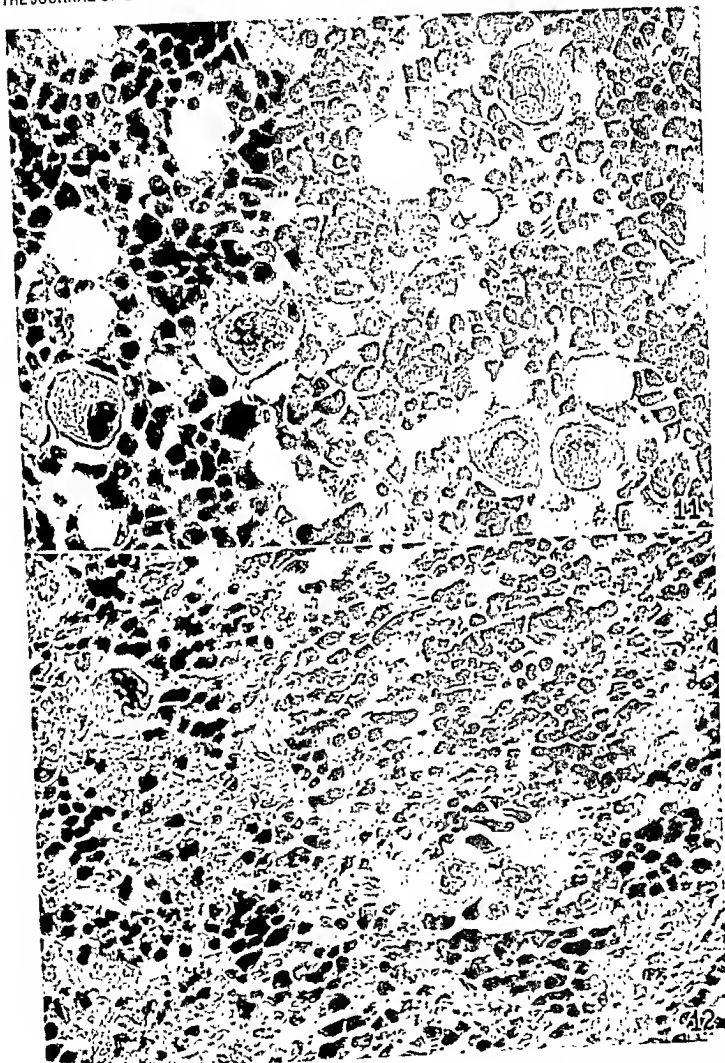
THE RELATION OF ALTERED LOCAL TISSUE REACTIVITY (SHWARTZMAN PHENOMENON) TO INFECTION AND INFLAMMATION

By ALAN R. MORITZ, M.D.

(From the Institute of Pathology, Western Reserve University, and University Hospitals, Cleveland)

Shwartzman has described a phenomenon of induced local hypersusceptibility to bacterial filtrates which differs in several important respects from the Arthus phenomenon although it appears to belong in the general category of immune processes. Menkin has suggested that the Shwartzman reaction is non-specific and that it may be explained by the concentration of intravenously injected irritative substance in an area of inflammation produced by a preceding local injection of bacterial products. Menkin's explanation was supported by the histological studies of Karsner and Moritz which showed that a primary local inflammation results from the preparatory injection and that the histological differences between it and the secondary local inflammation as manifested by the positive Shwartzman reaction are more quantitative than qualitative. Gerber has taken exception to Menkin's view and pointed out that there is no parallelism between the potency of a bacterial product in preparing a tissue for the Shwartzman reaction and the capacity of that product to produce inflammation. Gerber's histological studies of the phenomenon led him to support Shwartzman's original concept "that the preparatory factor induces a state of vulnerability of the tissue which is not in the nature of a mere trauma, increased permeability of capillaries or inflammation, but which is probably due to some functional disturbance in the cells which requires a short incubation period for its appearance and which disappears rapidly." The exact nature of this local functional disturbance manifested by hypersusceptibility to injury is still obscure. It was felt that whatever its nature may be, the effect of the local state of altered

reactivity on the survival, growth, and localization of pathogenic bacteria should be studied. Rich has recently reviewed the relation of the true allergic inflammation of hypersensitivity to the growth and spread of bacteria in man and lower animals, and he believes that there is no specific correlation between the degree of hypersensitivity and the degree of resistance to infection. He called attention to the fact that in the truly hypersensitive (allergic) body, not only the tissue cells but also the phagocytes are less resistant to injury, so that allergic inflammation *per se* constituted a lessened, rather than an increased, defensive mechanism.

A study of the relation of the Shwartzman phenomenon to resistance to and localization of infection was undertaken in two groups of experiments.

Methods

In the first group of tests, rabbits were inoculated subcutaneously with living organisms, and 24 hours later they were injected intravenously with a filtrate of the same organisms. These experiments afforded an opportunity to make histological and bacteriological observations as to the effect of the intravenous injection of filtrates on the resistance to and growth of bacteria at the site of primary infection.

In the second group of experiments, the rabbits were injected locally with bacterial filtrate and 24 hours later they were injected intravenously with living organisms. These experiments were carried out to determine the relation of the increased local vulnerability (produced by the local injection of bacterial filtrate) to the localization and subsequent growth of circulating bacteria.

A culture of *B. aertrycke* obtained from Professor E. E. Ecker was used for the preparation of bacterial suspensions and filtrates. Bacterial suspensions were prepared from the 24 hour surface growths on dextrose agar slants. The surface growth of each tube was washed off with sterile normal saline, shaken vigorously with glass beads, and filtered through No. 2 Whatman paper, and then centrifuged and washed and resuspended to the original volume of 2 cc. per tube. Bacterial filtrates were prepared from the 24 hour surface growth of organisms on Kolle flasks. 24 hours after inoculation the surface growth of each flask was scraped off and suspended in sterile distilled water (2 cc. per flask). The suspended organisms were passed through a Seitz filter and the filtrate was found to be potent as a subcutaneous preparatory factor for the Shwartzman reaction in amounts as low as 0.1 cc. without concentration (9 of 12 rabbits tested). Blank fluids prepared in the same manner as the suspensions and the filtrates, but without bacteria, were tested and were not potent either as preparatory or as provocative factors.

*Experiment 1. The Effects of Intravenously Introduced Bacterial
Filtrate on a Local Inflammation Produced by Homologous
Organisms*

Living organisms were substituted for bacterial filtrate to produce
a locus of increased vulnerability (Shwartzman phenomenon), and
24 hours after the local inoculation a filtrate from the same organism
was injected intravenously.   Alterations in the local reaction attribu-
table to the intravenous injection of bacterial filtrate were studied
morphologically and bacteriologically.

Pathological Study.—12 rabbits were injected subcutaneously in the right upper
quadrant of the abdomen with 0.05 cc. of a suspension of living *B. aertrycke.*
They were divided into three groups of 4 each. Rabbits 1 to 4 received no fur-
ther treatment and served as controls. Rabbits 5 to 8 received an intravenous
injection of 2 cc. of a potent[1] meningococcus filtrate 24 hours after inoculation
and also served as a control group. Rabbits 9 to 12 constituted the experimental
group and received an intravenous injection of 2 cc. of a filtrate prepared from
the same strain of *B. aertrycke* as was used for the subcutaneous inoculation.
All 12 rabbits were examined periodically and were killed by fracture of the
cervical spine at the intervals indicated in Table I.

A comparison of the gross characteristics of the local inflammatory
lesions in rabbits 1 to 4 with those of rabbits 5 to 8 showed them to be
similar and to consist of areas of subcutaneous edema of varying size
with or without perceptible hyperemia. The intravenous injection of
meningococcus filtrate in rabbits 5 to 8, 24 hours after inoculation with
living *B. aertrycke,* did not appear to effect any changes in the reactions
at the sites of inoculation   In rabbits 9 to 12 there was a striking gross
change in reactions at the sites of inoculation after the intravenous
injection of *B. aertrycke* filtrate. Before the intravenous injection of
filtrate, the local reactions were similar to those seen in the control
animals (Nos. 1 to 8) but within 4 to 6 hours after the intravenous

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[1] The meningococcus filtrate was potent as a preparatory factor in amounts of
0.5 cc. or more in 7 of 10 rabbits tested, if the rabbits received a subsequent
intravenous injection of meningococcic filtrate. The meningococcic filtrate was
not potent, however, in preparing the skin of the rabbit that was subsequently
injected with the *B. aertrycke* filtrate or in eliciting a positive Shwartzman reaction
in a rabbit prepared by the local injection of *B. aertrycke* filtrate.

TABLE I

Rabbit No.	Subcutaneous injection of 0.05 cc. living B. aertrycke	External examination of reaction at site of inoculation after 24 hrs.	Intravenous injection	External examination of reaction at site of inoculation Time after inoculation				Killed Time after inoculation
				30 hrs.	48 hrs.	72 hrs.	96 hrs.	hrs.
1	++	Edema	None	Edema				30
2	++	"	"	"	Edema			48
3	++	"	"	"	"	Edema		72
4	+	"	"	"	"	"	Edema	96
5	+	"	2 cc. meningococcus filtrate	"				30
6	++	"	"	"	Edema			48
7	++	"	"	"	"	Edema		72
8	+	"	"	"	"	"	Edema	96
9	+	"	2 cc. aertrycke filtrate	Hemorrhage and edema				30
10	+	"	" "	" "	Hemorrhage and edema			48
11	+	"	" "	" "	" "	Hemorrhage and edema		72
12	+	"	" "	" "	" "	" "	Hemorrhage and edema	96

injection, the local lesions became diffusely hemorrhagic with sub-cutaneous necrosis and extension of the area of edema.

Specimens for microscopic examination comprised the entire thickness of the abdominal wall and included the entire site of the reaction to inoculation. The tissues were fixed for 48 hours in 10 per cent neutral formalin, sections were cut at 6 micra and stained with hematoxylin-eosin and eosin-methylene blue.

Microscopic examination of the inoculated sites of rabbits 1 to 8 showed diffuse and dense infiltration of polymorphonuclear leucocytes at the end of 30 hours. This was associated with swelling and disintegration of collagen, edema, and a collection of fibrin in tissue spaces. At the end of 48 hours the lesions were more sharply circumscribed and active fibroblastic proliferation was apparent at the periphery. Necrosis of collagen was seen in the center and although many blood vessels became packed with white blood cells, very few vessels appeared thrombotic, and hemorrhage was inconspicuous. The organization became progressively more extensive and after 72 and 96 hours inflammatory lesions were definitely encapsulated. From the beginning there was marked degeneration and necrosis of exudative cells and there was a relative increase in the number of lymphocytes and large mononuclear cells as the interval after inoculation lengthened.

Bacteria were abundant in all specimens examined. 30 hours after inoculation large masses of intact organisms were seen in the tissue spaces. Many of these showed no penetration by leucocytes. With the passage of time, these solid clumps of organisms became dispersed and as nearly as could be judged from the eosin-methylene blue stained preparations, a large proportion of the bacteria were granular. Intracellular bacteria and basophilic granular masses were seen in both polymorphonuclear leucocytes and macrophages.

The inflammatory lesions in rabbits 9 to 12 differed from those seen in the control group (Nos. 1 to 8) in four important characteristics. Following the intravenous injection of homologous bacterial filtrate, the inflammatory lesions became hemorrhagic, extensive thrombosis occurred, there was more extensive fixed tissue necrosis, and organization was delayed. Whereas in the control group, small extravasations of erythrocytes were seen in the central portions of some of the lesions, diffuse central and peripheral hemorrhage was the rule in the experi-

mental animals. Large and small arteries and veins were thrombotic and their walls were frequently necrotic. A zone of necrotic skeletal muscle and fibrous connective tissue could be identified outside of the zone of active exudation. In contrast to the localization of the lesion after 72 and 96 hours, as described in the control group, there was very little evidence of encapsulation in comparable animals of the

TABLE II

Rabbit No.	Local inoculation of 0.05 cc. of suspension of living *B. aertrycke*	External examination of reaction at site of inoculation after 24 hrs.	Intravenous injection of *B. aertrycke* filtrate	External examination of reaction at site of inoculation after 48 hrs.	Colonies in millions per gram of tissue at site of inoculation Aliquot		
					A	B	C
	Suspension No.						
13	4-25	+ Edema	None	+ Edema	24.2	13.6	21.0
14	"	+++ "	"	+++ "			
		+ Hemorrhage		++ Hemorrhage	96.0	124.0	90.3
15	"	+++ Edema	"	+++ Edema	14.3	16.2	20.9
16	"	++ "	"	+++ "	26.1	22.4	20.8
17	"	++ "	2 cc.	+++ "	60.8	54.4	
18	"	++ "	" "	+++ "	184.6	192.1	207.0
				+++ Hemorrhage			
19	"	+++ "	" "	+++ Edema	240.0	232.1	186.2
				+++ Hemorrhage			
20	"	+++ "		(Dead in cage)			
21	6-26	++ "	None	+ Edema	12.3	8.4	14.2
22	"	+ "	"	+++ "	(Unsatisfactory plates)		
23	"	+++ "	2 cc.	+++ "	(Too many to count)		
				+++ Hemorrhage			
24	"	++ "	" "	+++ Edema	93.7	67.4	69.6
				+++ Hemorrhage			

experimental group. In the experimental group, the process, even at the end of 96 hours, was more of the nature of a phlegmon than an abscess. There were no significant differences between the cytological characteristics of the exudates of the two groups. In both groups, there was early degeneration and necrosis of exudative cells. In both groups, bacteria were numerous and a large proportion of them were granular.

Bacteriological Study.—Each of 12 rabbits was inoculated subcutaneously in the right upper quadrant of the abdomen with 0.05 cc. of a suspension of living *B. aertrycke*. The 12 were not all inoculated at the same time or with the same suspension of organism. 8 rabbits were inoculated with suspension 4-25 and four with suspension 6-26. These two suspensions were prepared in the same manner but the number of organisms was not determined. Although all of the animals of a group injected with the same suspension received approximately the same number of organisms, the number of organisms per inoculation in rabbits injected with suspension 4-25 was not known to be the same as that for rabbits injected with suspension 6-26.

Each group was divided into two subgroups of control and experimental animals, as is indicated in Table II. The control animals of each group received no further treatment. 24 hours after inoculation each of the experimental animals received an intravenous injection of 2 cc. of *B. aertrycke* filtrate and 24 hours later all control and experimental animals were killed by the intravenous injection of air. The skin of the abdomen was scrubbed with green soap and water, dried, scrubbed with ether, and then with 70 per cent alcohol. A block of tissue measuring approximately 1 x 1 x 0.5 cm. was excised from the central portion of the inoculated site, and was placed immediately in a sterile weighing bottle. The tissue specimens weighed between 1 and 1.5 gm. After weighing, the tissues were transferred to a sterile mortar and ground to a homogeneous pulp[2] with fine sand. The entire content of the mortar was transferred to a shaking bottle containing about 50 glass beads and 100 cc. of sterile normal saline. The bottles were shaken vigorously; specimens from rabbits 1 to 8 in a mechanical shaker for 20 minutes, and specimens from rabbits 9 to 12 by hand for 10 minutes. Dilutions of 1:10,000, 1:100,000, and 1:1,000,000 of the original mass of tissue were made and three plain agar plates were inoculated with 1 cc. from each dilution. A different aliquot of the original suspension of tissue was used for each plate. Colony counts were made at the end of 24 hours and 48 hours and the number of organisms per gram of tissue was calculated from these counts.

Although the results of these experiments were not entirely consistent, they afforded some data of interest when compared with the previously described pathological study. Of the 8 rabbits injected subcutaneously with suspension 4-25, 4 received a subsequent intravenous injection of bacterial filtrate. 3 of these survived and 2 of

[2] The epidermis resisted the most rigorous grinding and remained as a thin tough membrane. Microscopic examination of this membrane showed that it consisted of shreds of collagen fibers and epithelial cells. Since all specimens included about the same superficial area of skin and were ground for approximately the same length of time and with the same vigor, this resistant membrane was regarded as a fairly constant error.

these 3 had many more organisms per gram of tissue at the site of inoculation 48 hours after inoculation than did any one of the 4 controls. Rabbits 21 to 24 were injected subcutaneously with suspension 6-26 and of these, 2 received a subsequent intravenous injection of bacterial filtrate. Both of these had many more organisms per gram of tissue at the site of inoculation than did the one satisfactory control.

As has been already described in the pathological study (Table I), the inflammation became considerably more severe at the site of inoculation in the rabbits that received an intravenous injection of homologous bacterial filtrate 24 hours later. In the rabbits used in this section of Experiment 1 (Table II), none of the control rabbits, with the exception of rabbit 14, showed more than edema at the site of inoculation. 4 of the 5 rabbits that survived the subsequent injection of homologous bacterial filtrate showed a marked increase in severity at the site of inoculation and the bacterial counts indicated that this increase in severity of the local inflammation was, as a rule, associated with a relative increase in the number of living organisms at the site of inoculation as compared with similar determinations in rabbits of the control group.

Experiment 2. The Effect of Intravenously Introduced Organisms on Tissue Rendered Locally Vulnerable by a Preliminary Injection of Homologous Bacterial Filtrate

A locus of augmented vulnerability was produced by the subcutaneous injection of a bacterial filtrate and 24 hours later living organisms were substituted for bacterial filtrate as an intravenous injection to elicit the Shwartzman phenomenon. Alterations in the local reaction attributable to the intravenous injection of living bacteria were studied morphologically and bacteriologically.

As indicated in Table III, 16 rabbits were injected subcutaneously in the right upper quadrant of the abdomen with 0.2 cc. of the same preparation of filtrate prepared from *B. aertrycke*. Rabbits 25 to 32 served as controls. Rabbits 25 to 28 received no further treatment after the subcutaneous injection of filtrate. Rabbits 29 to 32 received an intravenous injection of 2 cc. of *B. aertrycke* filtrate 24 hours after the subcutaneous injection. Rabbits 33 to 40 constituted the experimental group and received an intravenous injection of 2 cc. of a suspension of living *B. aertrycke* 24 hours after the subcutaneous injection of filtrate. All

surviving animals in both groups were killed by air embolism 48 hours after the subcutaneous injection of filtrate.

Pathological Study.—Rabbits 25 to 28 showed no detectable changes on external examination at the end of 24 and 48 hours. 48 hours after injection, incision of the skin and subcutaneous tissue at the site of injection showed a mild degree of local edema. On microscopic examination there was sparse infiltration, principally by mono-

TABLE III

Rabbit No.	Subcutaneous injection of *B. aertrycke* filtrate	External examination of local reaction 24 hrs. after subcutaneous injection	Intravenous injection 24 hrs. after subcutaneous injection	External examination of local reaction 48 hrs. after subcutaneous injection
25	+	None	Not given	None
26	+	"	" "	"
27	+	"	" "	"
28	+	"	" "	"
29	+	"	2 cc. *B. aertrycke* filtrate	(Dead in cage)
30	+	"	" " " " "	Hemorrhage and edema
31	+	"	" " " " "	" " "
32	+	"	" " " " "	" " "
33	+	"	2 cc. living *B. aertrycke*	(Dead in cage)
34	+	"	" " " "	" " "
35	+	"	" " " "	Hemorrhage and edema
36	+	"	" " " "	" " "
37	+	"	" " " "	" " "
38	+	"	" " " "	" " "
39	+	"	" " " "	" " "
40	+	"	" " " "	" " "

nuclear cells, slight swelling of collagen and no more hemorrhage than could be attributed to the needle-puncture.

Rabbit 29 died about 2 hours after the intravenous injection of bacterial filtrate, remained in the cage overnight, and although there were no macroscopic changes at the site of subcutaneous injection, the tissue was not suitable for microscopic examination.

Rabbits 30, 31, and 32 all developed large hemorrhagic edematous lesions of the skin and subcutaneous tissue between 4 and 6 hours after

the intravenous injection. 48 hours after subcutaneous injection, there were present the characteristic microscopic changes of a positive Shwartzman reaction, which have already been described in detail by Karsner and Moritz, and Gerber.   Hemorrhage, venous and arterial thrombosis, necrosis, and diffuse and dense infiltration by polymorphonuclear leucocytes and large and small mononuclear cells were the outstanding microscopic characteristics.

Rabbits 33 to 40 received 2 cc. of living *B. aertrycke* intravenously 24 hours after the subcutaneous injection of bacterial filtrate.   Rabbits 33 and 34 died very soon thereafter, were found in their cages 8 hours later, showed no significant macroscopic changes, and were not

TABLE IV

Rabbit No.	Shwartzman reaction Aliquot			Croton oil reaction Aliquot			Arthus reaction Aliquot			Normal skin Aliquot		
	A	B	C	A	B	C	A	B	C	A	B	C
35	2.3	2.6	1.6	16.0	19.2	13.7				0.5	0.2	0.3
36	6.3	4.0	8.1	TM	TM	TM				0	0	0
37	20.3	19.2	11.3	30.0	28.5	16.5				0	0	2.9
38	31.3	38.7	34.4	68.0	72.9	79.7				4.3	5.4	4.5
39	22.5	15.7	43.6				4.1	5.3	3.9	1.2	3.3	1.8
40	11.0	18.8	14.3				7.4	8.3	6.5	2.4	1.6	5.7

Colonies in thousands per gram of tissue at site of:

TM = Too many to count.

suitable for microscopic examination.   Rabbits 35 to 40 were killed by air embolism 48 hours after the subcutaneous injection of filtrate and 24 hours after the intravenous injection of living organisms.   Except for the presence of bacteria in the exudate, the reactions were similar both in gross and microscopic features to those seen in rabbits 30, 31, and 32.   The living organisms served as a potent reacting factor in place of bacterial filtrate but did not produce a qualitatively or quantitatively different type of local reaction than was elicited by bacterial filtrate, except that bacteria did localize in the area of inflammation.

Bacteriological Study.—The number of living *B. aertrycke* that localized at the site of increased vulnerability were determined in the same manner as described in Experiment 1. In this experiment, however, it was necessary to

make much lower plating dilutions than were made in Experiment 1 because fewer organisms were present in the tissue. Each plate contained 1 cc. of dilutions of macerated tissue varying from 1:100 to 1:10,000 of the original tissue mass.

As indicated in Table IV, a sample of skin and subcutaneous tissue from a normal site (left lower abdominal quadrant) was taken for a control in each case. In each instance where such determinations were made (rabbits 35 to 40) there were many more bacteria at the site previously rendered vulnerable by an injection of a homologous bacterial filtrate than there were in normal skin and subcutaneous tissue from the same animal.

This finding could not be construed as evidence of the specific localization of bacteria because of a state of selective vulnerability induced by the injection of a homologous filtrate. To determine whether the presence of the bacteria represented a selective state of vulnerability or not, other types of inflammation were developed concurrently with the Shwartzman reaction in the same animals, and the number of bacteria present in the non-related sites of inflammation was determined.

In rabbits 35 to 38, 0.2 cc. of croton oil was injected subcutaneously in the right lower quadrant of the abdomen at the same time that the bacterial filtrate was injected in the right upper quadrant. 48 hours later, all of these animals had developed areas of acute hemorrhagic inflammation at the sites of croton oil injection, as well as at the site of the positive Shwartzman reaction. Determinations of the number of bacteria in the tissue in the regions of chemical inflammation revealed an even greater localization of bacteria there than at the sites of the positive Shwartzman reactions.

Rabbits 39 and 40 had been sensitized to horse serum 4 weeks before the experiment. The rabbits had received 4 subcutaneous injections at intervals of 4 days, consisting of 0.5, 0.5, 0.75, and 1.0 cc. of horse serum. At the same time that the bacterial filtrate was injected subcutaneously in the right upper quadrant, 0.5 cc. of horse serum was injected subcutaneously in the right lower quadrant. Both rabbits developed a large area of non-hemorrhagic subcutaneous edema (Arthus reaction) which, however, was not as severe an inflammatory response as was present at the site of the concomitant Shwartzman reaction. Determinations of the number of bacteria present at the site of the Arthus reactions revealed fewer organisms than were present in tissue from the positive Shwartzman reactions, but more organisms than were present in normal control skin from the same animals.

Living organisms served as a potent reacting factor when a homologous bacterial filtrate had been used to create a local site of increased vulnerability. The organisms tended to localize at the site of the positive Shwartzman reaction. This localization, however, was not specific inasmuch as bacteria localized in the same animals and in greater numbers in a locus of entirely unrelated inflammation (croton oil). Bacteria localized in greater numbers in the tissue at the site of the Shwartzman reaction than in tissue taken from a concomitant Arthus reaction but in these 2 animals the Arthus reaction was a less extensive and less destructive inflammation than was the concomitant Shwartzman reaction.

DISCUSSION

The demonstration that, in the case of *B. aertryckc*, the subcutaneous inflammation coincident with local infection in rabbits can be greatly modified and augmented by the intravenous injection of a filtrate of that organism 24 hours later, raises a number of questions which are not elucidated in this investigation. There was gross and microscopic evidence that inflammation became more severe at the site of inoculation as a result of the subsequent intravenous injection of homologous bacterial filtrate and that organization and repair were delayed. Such was to be expected since the positive Shwartzman reaction was added to the inflammation caused by the infection. Determinations of the number of living bacteria at the site of inoculation indicated that this augmentation of the severity of the inflammation furthered rather than inhibited their growth. On first consideration this appears to be in disagreement with the findings of Rivers and Tillett, Mallory and Marble, Opie, and Menkin, who have demonstrated that a preceding local inflammatory reaction tends to retard the dissemination of organisms from that site. Rich, however, has called attention to the fact that there is a great difference between implanting bacteria into a previously prepared exudate and implanting them in normal tissue. In these experiments the inflammation incident to the Shwartzman reaction followed rather than preceded the infection and the results are in accord with Rich's findings after the injection of fowl cholera bacilli together with pneumococcus lysate into the skin of hypersensitive (to pneumococcus lysate) animals. He found that there was

earlier dissemination from the site and more rapid death of the animals than in the case of controls in which the same dose of bacilli was injected without the concomitant injection of pneumococcus lysate. It was not determined in the present investigation whether a sub-lethal inoculation could be rendered lethal by the subsequent injection of homologous filtrates or not. It was not determined whether bac-teria entered the blood stream earlier from the area of inflammation because of the intravenous injection of the filtrate. It was not deter-mined whether local resistance was actually lessened by the accom-panying inflammation or whether it was only delayed. These and other questions deserve investigation.

In the second series of experiments it was shown that the subcu-taneous injection of bacterial filtrates led to increased local suscepti-bility to injury, so that when homologous organisms were injected 24 hours later, not only did an acute necrosing hemorrhagic inflamma-tion (Shwartzman reaction) develop locally, but bacteria tended to localize in the inflamed areas. This localization of homologous organisms was not a specific selective phenomenon but occurred also in concomitant sites of chemical (croton oil) and anaphylactic (Arthus) inflammation. The localization of bacteria at the site of the positive Shwartzman reaction is probably the same type of phenomenon as was observed by Menkin in the localization of intravenously injected graphite particles, *B. prodigiosus* or *B. pyocyaneus*, at the site of un-related subcutaneous inflammation. He felt that such a *locus minoris resistentiae* was due to the "increased permeability of capillaries with resulting accumulation and fixation of bacteria at the point of injury." In the case of the Shwartzman reaction, a site of potentially diminished resistance is created by the preliminary injection of filtrate (or or ganisms) which is converted into a real *locus minoris resistentiae* b the subsequent introduction of homologous organisms into the bloo stream. The organisms carry with them the agents responsible f the local inflammation, thus predisposing that site to their localizatio and growth. As in the case of the first series of experiments, the also suggest further lines of inquiry. Among these would be t substitution of living bacteria or bacterial filtrate in the provocati injection in the production of a generalized Shwartzman reactio It would be interesting to know whether the *in vivo* perfusion of org

with bacterial filtrate would lead to selective localization and growth of organisms subsequently introduced into the blood stream. In the experiment here reported, it was found that bacteria localized at the site of preparation with bacterial filtrate, but it was not learned whether they continue to grow at that site, or if the development of the inflammatory lesion is in any way altered because of the presence of the bacteria. These and other questions merit investigation.   .

CONCLUSIONS

Living *B. aertrycke* were effective in place of *aertrycke* filtrate as either the preparatory or the provocative injection in the production of a positive Shwartzman reaction.

When living *B. aertrycke* were injected subcutaneously a more severe inflammation resulted, organization was delayed, and more living organisms survived at the site of inoculation in rabbits that had received 24 hours later an intravenous injection of *B. aertrycke* filtrate than was the case in similarly infected rabbits that had not received a subsequent injection of bacterial filtrate.

When a local state of hypersusceptibility was created by the subcutaneous injection of *B. aertrycke* filtrate, the subsequent (24 hours) injection of living *B. aertrycke* led to the development at the site of subcutaneous preparation of a hemorrhagic necrosing inflammation in which the bacteria localized.

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THE BACTERICIDAL ACTION OF HUMAN SERUM ON HEMOLYTIC STREPTOCOCCI

III. Studies Concerning: (1) the Significance of Hydrogen Ion Concentration in Relation to the Streptococcidal Action of Serum; (2) the Effect of Reducing Agents on the Phenomenon

By WILLIAM S. TILLETT, M.D., and C. CHESTER STOCK,* Ph.D.

(From the Biological Division, Department of Medicine, Johns Hopkins Medical School and Hospital, Baltimore)

In previous reports[1,2] results were presented which demonstrated that sera from patients acutely ill with different infections were capable of destroying hemolytic streptococci of the *beta* type. As measured by the methods which were employed for estimating the streptococcidal action, comparable tests performed with sera obtained from the same group of patients very soon after they had recovered, revealed the loss of the property responsible for the lethal effect. Furthermore, by the same technique, normal sera were found to be essentially devoid of streptococcidal action. In the selected patients whose sera were used in the studies, the findings indicated that the serological property responsible for the bactericidal action was evoked or greatly increased by acute infections but that when the stimulus of active disease was no longer present, the killing action of serum for hemolytic streptococci rapidly subsided in a few days to a minimal level characteristic of normal serum. It was noted, in addition, that the streptococcidal potency was greatest in samples of sera derived from patients with the most severe types of illness.

The observations were made with three different strains of hemolytic streptococci which were selected for the investigation because they

differed in their sensitivity to the lethal effect of patients' sera.   One strain (Sc) was uniformly highly susceptible; a second strain (Ba) was affected only by the most potent samples of sera; the third strain (Co) was intermediate between the other two.   A subsequent report will describe other characteristics of a large number of strains in relation to susceptibility to sera.   At the present time it may be stated that, although there were exceptions, strains freshly isolated from patients having very severe types of infection have usually proven to be most vulnerable.

In addition to factors with respect to the activity of serum in relation to the course of the disease, and the selection of test strains, and the quantitative proportions of serum and number of bacterial cells used in the experiments,[1] the phenomenon was further characterized by the fact that the availability or exclusion of air in performing the tests had a marked influence on the viability of the organisms.[2]   It was demonstrated that when the serum-streptococcus mixtures were incubated anaerobically the killing action of patients' sera was markedly impaired or inactivated.   This finding appeared to be of possible significance in relation to the underlying mechanism of the phenomenon, and suggested that factors pertaining either to the aerobic-anaerobic physiology of the cells or to the oxidation-reduction state of the active principle in serum were important in determining the results.   In pursuing studies related to the nature of the streptococcidal phenomenon, additional data have been obtained which are contained in this report.

The findings to be presented deal with: (1) significance of hydrogen ion concentration in relation to the streptococcidal action of serum; (2) effect of reducing agents on the phenomenon.

Materials and Methods

The same experimental technique which was described in detail in the previous articles[1,2] has been regularly employed.   By recapitulation, 1.0 cc. of serum was inoculated with a platinum loopful of an 18 hour broth culture.   The full rounded drop used for inoculum contained approximately 800,000 to 1,000,000 organisms. The serum-streptococcus mixtures were kept in the water bath at 37.5°C.   Determinations of viability or destruction of streptococci were made by subcultures taken at such intervals during incubation as were indicated by the particular experiment; usually 6 hours and 24 hours were found to be most convenient and adequate.

Subcultures taken immediately after the addition of organisms to serum have regularly contained so large a number of colonies that actual count was impractical. Consequently when the number of colonies in subcultures was sufficiently reduced to permit actual estimations, the reduction in viable organisms was obviously considerable. It should be emphasized that the technical methods, which were used to measure the presence or absence of bactericidal activity in the specimens of sera, have certain limitations. For example, in any test in which the number of colonies in subcultures was maximum (∞), no quantitative differentiation was made, even though more detailed observations with appropriate dilutions would in all probability have revealed wide variations, referable to the extent of multiplication of the organisms above the number originally inoculated into serum. The results, obtained with normal sera and with other specimens inactivated by different methods although referred to as indicating the absence of killing power, are incomplete in accurate detail. In spite of the deficiencies just mentioned, the simple methods employed have served adequately in the studies of the serological property with which this series of articles deals.

Samples of sera were obtained from patients acutely ill with different kinds of infections. Since sera exhibiting a high degree of streptococcidal activity were usually required, preliminary tests of the samples were made, when possible, in order to insure satisfactory material. Consequently the patients from whom blood was obtained were selected on the basis of the severity of the infection. Specimens from cases of pneumonia have proven to be most regularly suitable and, for this reason, have been most frequently employed. When relatively large quantities of serum were required, the specimens from several different patients have been pooled.

One strain of hemolytic streptococcus of the *beta* type, designated Sc, has been used throughout the investigation. This strain is unusually highly sensitive to the streptococcidal action of serum and has given constant and uniform results.

Hydrogen ion determinations were made with Beckman's glass electrode pH meter which was calibrated daily by the use of accurately measured buffers. Other materials and methods will be described under the separate experiments.

Effect of Hydrogen Ion Concentration on Streptococcidal Action of Patients' Sera ·

As will be shown in the experiments which immediately follow, the hydrogen ion concentration of the specimens of sera may have, under certain conditions, a significant influence on the streptococcidal phenomenon and, conversely, the effect of the serum on the organisms may be followed by characteristic changes in the pH of the test material. The pH of serum is to a great extent conditioned by the CO_2 tension within it. In view of the fact that specimens, by giving off CO_2, continually progress toward equilibrium with the external air,

the pH increases in alkalinity until the liberation of CO_2 from the serum ceases. The rate of exchange of CO_2 is also influenced by the temperature, increasing within certain limits with increasing temperatures. Since the streptococcidal tests were done at 37.5°C. the thermal factor promoted the rate of change in pH which, as will be shown, required consideration. Data concerning this point will be given in the experiments which follow.

TABLE I

Influence of pH on Streptococcidal Action of Patients' Sera

(Tests performed under aerobic conditions)

Patient's serum No.	pH at beginning of experiment						
	*8.8–8.0	8.0–7.6	7.6–7.4	7.4–7.2	7.2–7.0	7.0–6.8	6.6–6.4
1	−†			−		−†	∞.
2	−	−		−			∞
3	−	−		−	−		∞
4	−	−	−	−		∞	
5	−	1†	32	90		∞	
6	−	−	23			∞	
7	−	2	1000	∞		∞	
8	35			∞			
9	200			∞			

* Represents range of pH in untreated sera. Other levels of pH obtained by addition of appropriate amounts of dilute HCl.

† Results of subcultures made after 24 hours incubation. − indicates no growth. Numerals indicate number of colonies by actual count. ∞ indicates innumerable colonies.

Number of colonies in subculture made at beginning of experiments was 1000s to ∞.

Numerous determinations of pH have been made on both normal and patients' sera, which have remained in the ice box, before being used in bactericidal tests, for periods of time from less than 1 day to several days. The results varied from pH 8.0 in the fresh samples to 8.8 in the older ones. These figures represent, therefore, the range of hydrogen ion concentration which was characteristic of sera at the beginning of the usual tests made under aerobic conditions.

In the first experiments concerning the effect of hydrogen ion concentration, the pH of samples of serum from patients was arbitrarily

adjusted to levels lower than those just mentioned. The results are given in Table I.

From the table it can be seen that adjustments of the hydrogen ion concentration in the acid direction had a marked effect on the killing power of sera. It should also be noted that abolition of the streptococcidal process did not occur at the same pH with each of the samples of sera. Repeated tests have indicated that inactivation of the active principle in sera of greatest potency requires a lowering of the pH to a greater degree than is necessary in order to produce the same effect in less active samples. For example (Table I), the streptococcidal activity of serum from patient 1 was maintained when the pH was decreased to 6.8–7.0 but was lost when the change was extended to 6.4. On the other hand serum from patient 9 (Table I) was active when tested without arbitrarily changing the pH (8.0+) but inactive when lowered to 7.4 –7.2.

It should be emphasized that the hydrogen ion concentrations recorded in Table I represent the pH at the beginning of the experiments. Because of the fact that changes in hydrogen ion concentration may occur during incubation, repeated measurements have been made in order to determine the level of pH in relation to viability of organisms. Sterile specimens of sera both from patients and normal individuals have been used as well as samples into which streptococci were introduced. The following protocol illustrates the results.

	Initial pH	Duration of incubation							
		2 hrs.		4 hrs.		6 hrs.		24 hrs.	
		pH	Subc.*	pH	Subc.	pH	Subc.	pH	Subc.
Normal serum, uninoculated............	8.15	8.70		8.90		9.03		9.42	
Normal serum + streptococci..........	8.15	8.42	∞	8.18	∞	8.46		9.15	∞
Patient's serum, uninoculated..........	8.21	8.78		8.92		9.04		9.42	
Patient's serum + streptococci........	8.21	8.80	800	8.83	500	9.04	220	9.39	16

* Number of colonies in subcultures.

From this experiment, which is representative of other comparable tests, it can be seen that, during incubation, there is a progressively increasing alkalinity in the uninoculated samples of both normal and patient's serum. The findings represent the characteristic changes

which occur in sterile serum during incubation at 37.5°C. It may be further noted that even in the test with patient's serum plus organisms the rise in pH was similar to that of the sterile specimens. However, the changes which occurred in normal serum containing streptococci did not parallel the findings obtained with the uninoculated sample, but continued at a lower level than the control tests for the first 6 hours of the experiment and, after 24 hours, had not risen to the degree found in the other tubes. The differences between the course of the changes in pH which occurred with the two samples of sera to which streptococci were added were secondary to the destruction, on the one hand, of the organisms by the patient's serum, and to their viability, on the other hand, in normal serum.

The limitations of the experimental methods which have been employed do not permit an exact correlation between the multiplication of organisms in normal serum and the changes in pH. However, it should be noted that the subculture taken after 24 hours incubation contained innumerable organisms even though the pH had risen to 9.15. In repeated tests with normal sera the final pH at the end of the experiment has been found to range from 8.8 to 9.2. With patients' sera which, on the other hand, caused extensive destruction of streptococci, the alkalinity after 24 hours rose to 9.2–9.5, the level regularly reached by uninoculated sera.

In view of the inconstancy of pH in the experiment just described, and since the final hydrogen ion concentration attained by patients' sera has been found to be relatively high even though organisms were added, additional tests have been carried out to determine the lethal effect exhibited by normal sera which had been adjusted to varying degrees of alkalinity.

	Initial pH	Subcultures		Final pH
		6 hr.	24 hr.	
Normal serum..........................	8.70	∞	∞	9.04
" "	8.90	∞	∞	9.10
" "	9.18*	∞	62	9.12
" "	9.29†	∞	90	9.39
" "	9.67†	1000s	20	9.64
" "	10.04†	1000s	48	9.72

* Specimen incubated 24 hours before inoculation.
† pH adjusted with NaOH.

From these results it can be seen that when the pH was maintained above 9.1 during 24 hours of incubation, evidence of considerable destruction of organisms was demonstrable in the subcultures taken at the end of the test period. The findings serve to indicate the pH at which normal serum exhibits a bactericidal effect that can be attributed to the degree of alkalinity.

When the results obtained with normal sera which were rendered toxic by raising the pH, are combined with the data derived from tests with patients' sera which were inactivated by lowering the pH, the effect of varying degrees of hydrogen ion concentration on the results may be estimated. The inactivation of patients' sera (Table I) occurred in the acid range at levels varying from 6.4 to 7.2–7.4, depending upon the potency of individual specimens. In the alkaline range of pH, some degree of toxicity was demonstrable with normal sera maintained for 24 hours above 9.1. It should be emphasized that the findings just described refer to the hydrogen ion concentration to which sera were adjusted at the beginning of the experiments. In view of the fact that in the tests with patients' sera, the pH gradually rose sufficiently high to contribute possibly to the final injury of the organisms, the effect of pH as a supplementary factor arising during the test period required consideration. The experiments which immediately follow bear upon this point. Methods were employed which were directed toward maintaining a relatively constant pH during the period of incubation.

Results Obtained under Conditions by Which Relatively Constant pH Was Maintained

In attempts to perform streptococcidal tests under conditions of fixed hydrogen ion concentration, difficulties have been encountered which appear to be dependent, to a considerable extent, upon adventitious effects introduced by the various methods which were tried. For example, when buffer solutions, such as phosphate, were added to serum, irregular results were obtained which were related to varying degrees of sediment precipitated from the mixtures.

Up to the present time a relatively constant pH has been most satisfactorily maintained by the use of jars in which definite tensions of CO_2 were established. By this means, the hydrogen ion concentra-

tions of the sera could be stabilized by equilibration with air containing a measured amount of CO_2.

The jars which were employed had a single outlet from the top. Through a rubber tube attachment, the air in the jar was partially evacuated. By preliminary determinations, the amounts of CO_2 necessary to obtain various levels of pH were measured by a mercury manometer connected to the outlet of the jar. For example, to maintain the pH of the serum at approximately 8.0–8.3 it was necessary to introduce into the evacuated jar sufficient CO_2 to register on the manometer an increase of pressure equal to 2.0 to 2.5 cm. of mercury. After the CO_2 was introduced, the pressure within the jar was finally raised to atmospheric level by permitting air to enter. The rubber tubing was then clamped. The jars containing the sterile sera, distributed in 1 cc. amounts in separate tubes, were kept overnight to allow equilibrium to be established. They were opened the next morning, the inocula of streptococci were introduced, and the jars were again prepared as described. The pH in one tube of sterile serum was measured in order to determine the hydrogen ion level at the beginning of the experiment. It is interesting to note that when specimens of sera from different patients were placed in the same jar, the pH's of the separate samples were not always the same following the preliminary 18 hour period allowed for adjustment. However, by placing increments of the same serum in several different jars adjusted with different quantities of CO_2, the activity of the serum could be estimated at different levels of pH.

When readings and subcultures were made at 4 or 6 hours it was again necessary to reopen the jars, make the tests, and prepare the jars for the subsequent incubation. These procedures were carried out with sufficient speed to minimize the duration of exposure to room atmosphere.

Although the method just described possessed definite limitations, the data gained from a number of experiments have been sufficiently satisfactory to be informative with respect to the significance of hydrogen ion concentration. However, it should be emphasized that the several manipulations which were necessary may, in some instances, have caused additional changes, the significance of which has not yet been established. Further studies, with the hope of perfecting the methods, are now in progress.

The results obtained with tests performed in closed jars are presented in Table II.

The observations indicate the ranges of alkalinity in which the streptococcidal action of sera was demonstrable. Because the amount of the individual samples of sera was necessarily small, it has not been possible to make a sufficient number of tests with one specimen at different levels of pH to define sharply the border line of hydrogen

ion concentration above which bactericidal action was demonstrable and below which no killing occurred. However, the results clearly

TABLE II

Streptococcidal Tests Performed in Closed Jars at Relatively Constant Levels of Hydrogen Ion Concentration

(Results compared with those obtained under aerobic conditions)

Patient's serum No.	Condition of test	Initial pH	4 hrs.		6 hrs.		24 hrs.		pH range during incubation	Bacterial power of serum*
			Subc.	pH	Subc.	pH	Subc.	pH		
1	Aerobic	8.32			8	9.02	—	9.28	8.32-9.28	++++
	Jar	7.95	240	8.20	130	8.22	—	8.19	7.95-8.22	++++
2	Aerobic	8.45			2	9.10	—	9.46	8.45-9.46	++++
	Jar	8.60			26	8.62	—	8.58	8.58-8.62	++++
	"	8.40			46	8.27	—	8.32	8.27-8.40	++++
	"	7.95			1000s	7.64	800	7.79	7.64-7.95	+
3	Aerobic	8.55			2		—	9.41	8.55-9.41	++++
	Jar	8.10			24	8.35	18	8.24	8.10-8.35	+++
4	Aerobic				64		—	9.46		++++
	Jar	8.57			150	8.44	80	8.48	8.44-8.57	+++
	"	8.24			∞	8.18	∞	8.09	8.09-8.24	—
5	Aerobic	8.62	240		60		—	9.35	8.62-9.35	++++
	Jar	8.32	500	8.31	250	8.24	120	8.43	8.24-8.43	++
6	Aerobic				800		16	9.32		++
	Jar	8.30	∞	8.23	∞	8.17	∞	8.20	8.17-8.30	—
7	Aerobic	8.47			800		15	9.26	8.47-9.26	++
	Jar	8.48	∞	8.21	∞	8.33	∞	8.20	8.20-8.48	—
8	Aerobic				260		3	9.27		+++
	Jar	8.57			∞	8.44	∞	8.48	8.44-8.57	—

For interpretation of figures see footnotes to Table I.

* ++++ indicates most active killing power. +++, ++, +, — are proportional ratings below maximal activity.

demonstrate that the level of pH at which the tests *in vitro*, were performed, had a significant influence on the streptococcidal property of sera. With the experimental technique which was employed to

keep a uniform pH during incubation, the bactericidal effectiveness of sera has been optimum at levels of hydrogen ion concentration above neutrality.

The findings recorded in Table II were obtained with specimens of serum which exhibited varying degrees of streptococcidal activity in the usual tests carried out under aerobic conditions.   The differences in potency were also evident from the results obtained under relatively constant conditions within jars, as indicated by the different levels of hydrogen ion concentration required to inactivate the killing power of different specimens of serum.   Serum 1, for example, remained potent in the range from 7.95 to 8.22.   Serum 8, on the other hand, was not actively bactericidal in the higher range of 8.44 to 8.57.

It is interesting to note that in the aerobic tests concerning the effect of pH (Table I), a decrease in the initial pH below neutrality was required to inactivate most of the samples of sera.   In the experiments cited in Table II, abolition of killing power was effected by retaining the pH at a higher level throughout 24 hours.   The apparent discrepancy appears to reside in the fact that in the aerobic tests the initial killing power of the serum was retained sufficiently well to exclude the lowering effect on pH of active bacterial metabolism. Consequently, the hydrogen ion concentration progressively increased and, as a result of the loss of CO_2, reached the optimal degree for the effectiveness of the streptococcidal principle, and may have even continued into the range of alkaline toxicity which was characterized in the tests made with normal serum at pH 9.1+.

Effect of Conditions of Reduction (Anaerobiosis and Reducing Agents) on the Streptococcidal Activity of Sera

In a previous report[2] it was demonstrated that the streptococcidal action of patients' sera was inactivated when the tests were carried out under the anaerobic conditions afforded by a vaseline seal or an anaerobic jar.   In pursuing the investigation of the possible influence of states of oxidation-reduction on the phenomenon, the effect of reducing agents on the killing power of sera has been tested.   As will be shown later in this article, definite results were obtained.   However, in considering the mechanism responsible for the effect, several factors need to be taken into account.   For example, since hydrogen ion concentra-

tion has been found to be significant, it has been necessary to make observations on the levels of pH in all the tests. For purposes of comparison, therefore, before presenting a description of the results obtained with chemical reductants, it is interesting to note the course of the changes in pH which occurred under the reduced state afforded by anaerobic conditions.

Significance of pH in Anaerobic Tests.—In view of the fact that the methods employed to obtain anaerobiosis also retarded the escape of CO_2 from serum, and for this reason prevented the usual increase in pH which occurred under aerobic conditions, anaerobic tests have been repeated with special consideration being given to hydrogen ion concentrations. The data derived from numerous preliminary observations indicated that, when sera were inactivated by layering with a column of vaseline, the number of viable organisms present in subcultures made after 24 hours incubation was always considerably greater than the number present in subcultures from the aerobic tubes, but that the pH of the material in the anaerobic tubes at the end of the test period varied from the initial point to lower levels in the acid direction. Since the reduction in pH might possibly contribute to the inactivation, more detailed observations have been made with other samples of serum in an attempt to evaluate, if possible, the separate significance of the anaerobic effect and changes due to pH.

From the data given in the following protocol, the changes in pH which occurred under aerobic and anaerobic conditions, may be compared.

	4 hrs.*		6 hrs.*		24 hrs*	
	pH	Subc.	pH	Subc.	pH	Subc.
Patient's serum,† uninoculated, aerobic.........	9.16		9.25		9.55	
Patient's serum + streptococci, aerobic.........	9.15	310	9.23	250	9.52	1
Patient's serum, uninoculated, anaerobic	8.57		8.56		8.55	
Patient's serum + streptococci, anaerobic.......	8.62	800	8.57	1000s	8.22	∞

* Duration of incubation.

† pH of serum at beginning of experiment: 8.50.

The results demonstrate the fact that the pH in the tubes kept under anaerobic conditions did not rise as in the aerobic tests. Interest

centered, therefore, around the question of whether the levels of pH in the sealed tubes were sufficiently low to account for the results on the basis of hydrogen ion concentration, *per se.*   A discussion of this aspect of the problem will be given later on in this article.

Effect of Reducing Agents on the Streptococcidal Activity of Serum

In continuing a study of the possible influence of oxidation and reduction on the bactericidal activity of sera, oxidizing and reducing agents have been added to test materials.   With respect to oxidizing

TABLE III

The Inactivating Effect of Glutathione and Sodium Ascorbate on the Streptococcidal Activity of Sera

Patients' sera No.	Without glutathione				With glutathione			Patients' sera No.	Without Na ascorbate			With Na ascorbate	
	pH of sera	Subcultures		Final pH	Subcultures		Final pH		pH of sera	Subcultures		Subcultures	
		6 hr.	24 hr.		6 hr.	24 hr.				6 hr.	24 hr.	6 hr.	24 hr.
1	8.22	600	7	9.60	∞	1000s	9.05	11	8.60	90	—	∞	∞
2	8.21	220	16	9.39	∞	600	8.90	12	8.72	160	50	∞	∞
3	8.40	13	—		∞	500		13	8.56	150	25	∞	∞
4	8.09	250	—	9.50	∞	400	8.83	14		125	2	1000s	∞
5		2	—		∞	320		15		48	—	1000s	1000s
6		800	5		∞	110		8		1	—	800	∞
7	8.92	250	6	9.48	600	250	8.88	5		2	—	250	1000s
8		1	—		300	2		16	8.50	20	—	220	1000s
9		1	—		100	15		17	8.85	12	—	76	1000s
10	8.58	140	2	9.48	180	56	9.17	9		1	—	—	15

For interpretation of figures, see footnotes to Table I.

substances, the results have been irregular and difficult to evaluate, due, to a great extent, to the toxic effects on the bacteria of the materials themselves.   Consequently, the findings, so far obtained, are too incomplete to justify presentation.   Studies on this phase of the problem, which are now in progress, will be subsequently reported.

In experiments in which reducing agents were added to sera, the most consistent and definite results have been obtained with glutathione and sodium ascorbate.   The results are given in Table III.

0.5 M solutions of each of the materials were made in sterile distilled water and always used immediately after preparation. 0.1 cc. of each was added to 0.9 cc. of serum and well mixed, making a final concentration of 0.05 M. Organisms were introduced within a few minutes after the reducing agents were added. The pH of the solutions was always adjusted to approximately 8.2 to 8.6.

From the data presented in Table III, it can be seen that, in the majority of instances, the bactericidal property of the specimens of sera was either completely inactivated or markedly impaired by a 0.05 M concentration of glutathione or sodium ascorbate. Although the difference between the number of viable organisms present in sub-cultures from the sera alone and from serum-reductant mixtures is striking, the results do not indicate whether inactivation was dependent upon the reducing property of the reagents or upon some other possible chemical reaction involving the active serological principle. The complexity of the serum-streptococcus mixtures and other difficulties have rendered exact physical chemical measurements unsatisfactory. Consequently, conclusive evidence supporting one or the other possibility cannot, at present, be given.

An analysis of the results indicates that the effect on serum of each of the reducing agents is, in general comparable. However, the results obtained with glutathione and with sodium ascorbate have, in some of the tests, suggested individual differences. For example, in the experiments in which glutathione was used, inactivation appeared to be complete during the first 6 hours of incubation, but was not sustained for 24 hours as evidenced by the decrease in number of colonies obtained in subcultures made at the termination of the experiment. Since glutathione in normal serum exerted no demonstrable toxic effect on the organisms, the findings with patients' sera plus glutathione suggest that the reducing material may be decomposed by the living organisms, or by auto-oxidation, and that the late killing effect depends upon the reactivation of the streptococcidal property following the alteration of the inactivating agent.

In the tests in which the nullifying effect of sodium ascorbate was only partial, the incompleteness of inactivation was demonstrated by the fact that although ultimate growth of organisms occurred after 24 hours, the final result was preceded by a temporary drop in viable bacterial population.

In considering the mechanism by which both glutathione and sodium ascorbate alter serum, the ability of both substances to form complex compounds requires consideration. For example, the double bond present in the structure of ascorbic acid offers a possibility of chemical combination in the serum-streptococcus mixtures which may influence the phenomenon. In view of the possibilities from the standpoint of diverse chemical reactions, therefore, a final interpretation of the process by which the reagents inactivate the streptococcidal principle will require more detailed analysis. The findings presented in this article demonstrate, however, that marked alterations in the serological property responsible for the bacterial destruction result from the addition of chemical reductants to the test material.

In spite of the several aspects of the problem just referred to, when the results obtained by adding reducing agents to sera are taken in conjunction with the inactivating effect of anaerobiosis previously described,[2] the findings in both instances strongly suggest that the streptococcidal action of patients' sera may be influenced by conditions of oxidation-reduction. In this connection it is also interesting to note that in the tests in which the effect of the reducing agents was least marked, the sera which were used were especially potent in killing action. In the previous experiments carried out anaerobically,[2] it was also found that specimens of sera of greatest potency were less completely inactivated by excluding air than were samples of moderate activity. A general parallelism between the results obtained with reducing agents and those derived from tests performed anaerobically has characterized all of the observations up to the present time.

In the experiments in which reducing substances were added to sera, the factor of hydrogen ion concentration was taken into consideration. By properly adjusting the pH of the reducing materials before addition to serum, the hydrogen ion concentration of the tests was always comparable at the beginning of the experiments. The subsequent changes in pH of the sera, with and without the added reductants, seemed to be dependent upon the secondary effect referable to the viability of the streptococci. A detailed correlation of pH and number of living organisms is illustrated by the following findings.

	2 hrs.		4 hrs.		6 hrs.		24 hrs.	
	pH	Subc.	pH	Subc.	pH	Subc.	pH	Subc.
Patient's serum, uninoculated........	8.78		8.92		9.04		9.42	
Patient's serum, uninoculated, + glutathione....................	8.36		8.46		8.53		9.10	
Patient's serum + streptococci......	8.80	800	8.88	500	9.04	220	9.39	16
Patient's serum + glutathione + streptococci....................	8.38	∞	8.37	∞	8.40	∞	8.90	600

pH at beginning of experiment: 8.2.

From the observations made with sterile specimens of serum plus glutathione it may be seen that the glutathione itself had a slight buffering effect on serum during incubation, since the pH in the uninoculated sample, containing the reductant, increased more slowly than did the control and did not eventually reach the degree of alkalinity attained by the sterile serum. It should be further noted that, in the glutathione-serum preparation to which streptococci were added, inactivation of the bactericidal factor was evident by the innumerable colonies obtained at each subculture for 6 hours. The pH during these periods did not, however, increase as in the control serum-streptococcus mixtures. The findings at 24 hours indicated that some killing had taken place, possibly occurring as previously mentioned, after decomposition of the glutathione, and that the pH had accordingly risen.

A limited number of aerobic experiments similar to that just described have been carried out using sodium ascorbate instead of glutathione. In the few instances in which hydrogen ion measurements have been correlated with survival of streptococci, the reductant has been found to exert a slight buffering effect, and the rise or fall in pH seemed to be conditioned by the number of viable organisms; subcultures of the serum-ascorbate mixtures contained several hundred to countless colonies, whereas in control tests, progressive destruction of the organisms occurred.

From an analysis of the findings with respect to the effect of anaerobiosis and of reducing agents, the problem arises as to whether the procedures which were used to obtain reduced conditions abolished the streptococcidal action of sera through inducing and maintaining a

level of hydrogen ion concentration below the range of bactericidal activity, or whether factors pertaining to biological reduction were involved in the inactivation.   In numerous attempts to gain information concerning the possibilities just mentioned, complexities have been encountered which have made an interpretation of the results difficult.   In some of the tests, however, the inactivating effect of reducing agents and of anaerobiosis was observed at the same level of pH at which the organisms were destroyed by unaltered samples of the same serum.   This was most often noted after periods of only a few hours incubation rather than after the complete 24 hours of the usual test.   The following results illustrate the course of the experiments.

		Duration of incubation			
	Initial pH	6 hrs.		24 hrs.	
		Subc.*	pH	Subc.	pH
Patient's serum, jar............................	8.61	120	8.72	300	8.60
Patient's serum + glutathione, jar..............	8.61	1000s	8.67	∞	8.49
Patient's serum, anaerobic......................	8.65	∞	8.71	∞	7.98
Patient's serum, jar............................	8.54	22	8.75	—	8.70
Patient's serum + glutathione, jar..............	8.54	500	8.71	∞	8.50
Patient's serum, anaerobic......................	8.62	400	8.85	∞	8.12

* Number of colonies in subcultures.

In the measurements made at 6 hours, it may be noted that the pH in each set of tubes is essentially the same, but that a greater number of viable organisms have been recovered from the serum-glutathione and the anaerobic tubes than from the untreated serum in the jar. After 24 hours, however, the discrepancies in pH are significant and may be an important contributing factor.   The fact that conditions which promoted the lowering of pH were uncontrolled has constituted one technical difficulty which makes an explanation of these results uncertain.

Although the observations just described are suggestive, it has been of interest to pursue further studies concerning this phase of the problem.   As the investigation has progressed, the results obtained during 24 hours incubation have seemed to be conditioned by several

factors which appear to be interdependent and each of which may vary during the prolonged test period. Contributing factors which require consideration are composed of, differences in the streptococcidal potency of individual samples of serum, differences in the range of pH at which samples of sera are primarily bactericidal, and effects referable to bacterial metabolism. Consequently, a final analysis of the mechanism, by which the additional factor contributed by oxidation-reduction affects the phenomenon, must await further study. Methods are, at the present time, being developed, by which both the individual factors and their quantitative relationships are more satisfactorily controlled. The findings will be subsequently reported in detail.

DISCUSSION

With respect to hydrogen ion concentration, the results have indicated the effect of pH on the streptococcidal activity of sera and have defined the progressive changes in pH which occurred in the tests in relation to the destruction of hemolytic streptococci by patients' sera, and to the maintenance of viability or growth in normal sera.

By arbitrarily lowering the hydrogen ion concentration of patients' sera, a degree of acidity was reached which inactivated the streptococcidal property. Conversely, by increasing the pH of normal sera, a level of alkalinity was attained which was injurious to the organisms. The intermediate zone between the two extremes represented the levels of hydrogen ion concentration most favorable for determining bactericidal action.

However, even in tests which were begun in ranges of hydrogen ion concentration that avoided the primary effects of pH, subsequent changes were found to occur under aerobic conditions during the period of incubation. When the organisms remained viable, their metabolic activity impeded the spontaneous rise in pH characteristic of sterile serum during incubation. When progressive destruction of the bacteria occurred, an increasing alkalinity developed such as that observed in control specimens of serum which were uninoculated with organisms. The course of changes in pH was found to be, therefore, primarily dependent upon the streptococcidal activity of sera. By making repeated estimations during the test period, the pH, in many instances, was found to be sufficiently changed so that the hydrogen ion con-

centration became a contributing factor in the effect of the serum on streptococci. With the rise in pH (9.2–9.5), which occurred during incubation in sera possessed of streptococcidal activity, a degree of alkalinity was reached, which, according to tests made with normal sera, may have contributed to the injurious effect of the former specimens.   On the other hand, the viability and probable multiplication of organisms in normal sera served to maintain the pH at lower levels favorable for their survival.

In the observations, therefore, made under aerobic conditions, in which the changes in pH were uncontrolled, the results illustrate the primary effect of the streptococcidal principle and the supplementary influence that may be introduced by the levels of hydrogen ion concentration.

In the light of the observations with respect to pH, it became necessary to control this factor in order to characterize more clearly the streptococcidal principle itself.   The conditions were reasonably well fulfilled in experiments carried out in closed jars into which measured amounts of CO_2 were introduced.   In spite of certain technical difficulties, the results have indicated the approximate ranges of pH at which the bactericidal property was active under the experimental conditions which were employed.   As in all the studies concerning the bactericidal activity of sera against hemolytic streptococci, differences in results were conditioned by the potency of individual specimens of serum.   In the present experiments, maintenance of streptococcidal activity was most marked only in alkaline ranges but the optimal level differed with individual specimens of serum.   The most potent samples of serum remained active at about pH 7.9–8.2.   With specimens of sera of less potent killing power, activity was evident when the pH was kept constant between 8.4 and 8.8.   It may be seen, therefore, that, with the special type of procedures employed in the tests, the streptococcidal activity of sera functioned best at alkaline levels but that the stronger the sample of serum the lower the pH could be adjusted without impairing the bactericidal effect.

As previously reported,[2] the inactivating effect of anaerobiosis on the streptococcidal phenomenon suggested the possibility that some factor relating to oxidation-reduction might influence the reaction.   In studies directed toward the possible rôle of the oxidation-reduction

potential numerous difficulties have been encountered pertaining to quantities and complexities of the biological materials used in the tests. Consequently direct and accurate physical chemical measurements have, up to the present time, not been satisfactory. Indirect evidence has been sought through the use of oxidizing and reducing agents, on the assumption that oxidation might enhance the bactericidal action which is effective under conditions of aerobiosis, and that a state of reduction might impair the activity in a manner comparable to the inactivating effect of anaerobiosis.

With respect to the effect of oxidizing agents, the materials so far tried have been found to possess some degree of bactericidal activity themselves. Consequently the data obtained up to the present time are too incomplete to warrant presentation. Further studies concerning this approach to the problem are now being pursued.

The results obtained when certain reducing substances, such as glutathione or sodium ascorbate, were added to serum demonstrated that these substances markedly impaired the streptococcidal activity of specimens of serum from patients in a manner comparable to that occurring anaerobically.

In view of the importance of hydrogen ion concentration, it was necessary to analyze the results obtained with reducing agents and with anaerobic conditions for the purpose of determining the extent to which pH contributed to the effect. In tests bearing upon this point the inactivating effect of states of reduction was observed, in some instances, at levels of pH at which bactericidal action took place in untreated increments of the same sera. However, in other comparable tests, an interpretation of the results has been uncertain, due to variations in the results which were dependent upon the limitations of the experimental methods which were employed. Further studies are now in progress which are directed toward a complete analysis of the rôle of biological oxidation-reduction in relation to the streptococcidal action of serum.

SUMMARY

Changes in pH Which Occurred during Incubation.—

Aerobic Tests. (a) The pH of sterile specimens of both normal and patients' sera increased, during 24 hours incubation, from 8.0–8.8 up to 9.2–9.6.

(*b*) The pH of patients' sera, inoculated with hemolytic streptococci, progressed in the alkaline direction as did the sterile specimens. (*c*) The pH of normal sera, inoculated with hemolytic streptococci, pursued a variable course at lower levels than the sterile specimens. The differences in the changes in pH which occurred in streptococcidal sera and in normal controls were dependent upon and secondary to the presence or absence of killing action in the specimens.

Aerobic Tests.   Effect of Different Levels of Hydrogen Ion Concentration, Adjusted at Beginning of Experiment, but Uncontrolled during Period of Incubation.—(*a*) The streptococcidal activity of patients' sera was inactivated when the hydrogen ion concentration of the specimens was adjusted to levels ranging from 6.4 to 7.4. The inactivation of highly potent samples of sera required a greater reduction in pH than did specimens of moderate killing activity. (*b*) Normal sera, adjusted to pH 9.2 or higher, exhibited a relatively slowly acting bactericidal process, which seemed to represent the toxic effect of alkalinity.

Aerobic Tests.   Effect of Glutathione and Sodium Ascorbate.—The streptococcidal action of patients' sera was markedly impaired by the addition of reducing agents. The inactivation was, in general, comparable to that previously obtained under conditions of anaerobiosis. The results, suggesting that some factor pertaining to biological oxidation-reduction may influence the streptococcidal action of sera, have been discussed.

Tests Performed in Closed Jars by Means of Which Relatively Constant pH Was Maintained during the Period of Incubation.—Under the experimental conditions which were employed, the effectiveness of the streptococcidal action of patients' sera was maintained only in alkaline ranges.   The most potent specimens of sera were active at lower levels of pH (7.9–8.2) than were other samples (8.4–8.8).

All of the observations reported in this article were made with a strain of hemolytic streptococcus of the *beta* type, which is highly sensitive to the destructive action of serum from acutely ill patients.

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CHANGES IN BLOOD VESSELS (CAPILLARY FRAGILITY) WITH INFLAMMATION

By ERNST ZANDER

(From the Department of Pathology, Cornell University Medical College and New York Hospital, New York)

The occurrence of hemorrhage in association with many forms of inflammation has suggested a study of changes in the vascular walls that favor the escape of red blood corpuscles. Inflammation of the cutis has been produced by a variety of means and the ability of the vessels to withstand mechanical injury produced by suction applied to the skin surface has been determined.

Capillary bleeding can be produced by altering the relation between extra- and intracapillary pressure. When venous flow is obstructed by application of an elastic bandage to the arm intracapillary pressure is increased and petechiae occur. Rumpel (1) and Leede (2) discovered this phenomenon in patients with scarlet fever. Goethlin (3), Bayer (4), Bexelius (5), Walterhoefer (6) and others have developed methods of measuring capillary fragility based on this phenomenon. Goethlin, Shultzer (7) and others found a marked increase in capillary fragility in patients suffering from scurvy and a somewhat increased tendency to capillary bleeding in patients who had a history of vitamin C deficiency in their diet, as in patients fed on a diet of milk, cream and cereals used in the treatment of peptic ulcer. Walterhoefer found that the fall upon the forearm of a weight of 130 gm. from a height of 35 cm. increased the number of petechiae which occur when an elastic bandage is applied to the arm. Bayer obtained an increased number of petechiae when he applied linen saturated with hot water for 5 minutes before application of the elastic bandage. He described increased capillary fragility in skin areas of slight erythema produced by ultraviolet light. Bexelius found the number of petechiae somewhat increased when the patient took a hot steam bath shortly before he applied the elastic bandage.

Hecht (8), Silva Mello (9), Dalldorf (10) and Cutter (11) produced capillary bleeding by application of a partial vacuum to the skin. They used this suction test to recognize changes in the blood vessels with vitamin C deficiency. They determined the lowest partial vacuum which suddenly applied to the skin produced petechiae, and counted their number.

In the following experiments, which have been done under the direction of Dr. E. L. Opie, suction is used as a measure of the injury required to produce hemorrhage. A vacuum applied to the skin of rabbits is slowly increased till the pressure reaches -70 mm. Hg and this level is maintained until a uniform dark red hemorrhage composed of innumerable petechiae has occurred over the whole area subjected to the suction. The time necessary to produce this effect is an approximate index of susceptibility of the capillary wall to hemorrhage, designated for convenience capillary fragility.

Fig. 1 shows the suction apparatus that has been used. It consists of a water pump, two 2000 cc. glass bottles (I and II), a 500 cc. separator (III), a mercury manometer and a glass tube to apply the suction to the skin, all connected by means of heavy walled rubber tubes and connecting glass tubes. A high partial vacuum is made in the reservoirs I, II and III by means of a water pump. A number of stop-cocks transfer this pressure slowly or suddenly to the suction tube that is applied to the skin at the desired point. The pressure in the reservoirs can be measured by the manometer by turning the 3 way cock D and opening stop-cock G. Stop-cock E is permanently adjusted so that air from the suction tube passes very slowly through it into vacuum reservoir III. By opening stop-cock F for about 1 minute the vacuum in the suction tube gradually increases until a pressure of -70 mm. Hg is reached. Continuity of the suction tube with vacuum reservoir I after stop-cock B is opened permits immediate production of vacuum in the suction tube. By opening stop-cock C air can be let into the suction tube and atmospheric pressure regained. The suction tube is a simple straight glass tube, 8 cm. long, 8 mm. in diameter and 1 mm. thick, carefully rounded at the end. Vaseline applied to the skin of the rabbit makes the junction between skin and tube air-tight. With the apparatus that has been described a vacuum applied to the skin remains at constant pressure for at least 1 hour.

The hair of the rabbit has been clipped 24 hours before testing because shaving produces strong irritation of the skin. Rabbits weighing more than 2000 gm. have been used. Suction with gradually increasing vacuum produces gradually increasing hyperemia of the normal skin. When the suction test is applied to smaller rabbits with thin skin the hyperemia occasionally fails to occur and the observations are unsatisfactory. It is found empirically by applying suction to the skin in several places on the same animal that a vacuum of -70 mm. Hg gives more constant results than suction with higher or lower pressures.

With normal skin of the rabbit petechiae usually appear after 4 to 5 minutes of suction and in most rabbits hemorrhage occurs after 8 minutes over the whole area subjected to the suction. There are only slight differences in the results of the test applied to skin in different
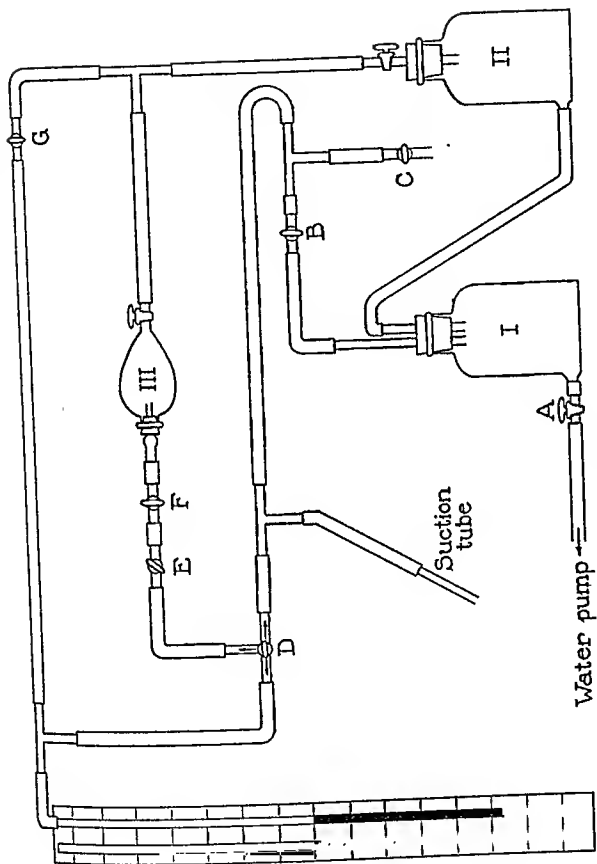
Fig. 1

places from the spine to the mammillary line. The suction provokes capillary bleeding more quickly when it is applied adjacent to previously made hemorrhages and tests should be made at least 3 cm. apart. Suction time in subsequent experiments is the time required to produce the vascular injury that is revealed by hemorrhage.

Suction causes hemorrhages into the skin of the legs after an interval of approximately 2 or 3 minutes longer than that required for their production on the flank of the animal. Hemorrhages in the skin of the ears are produced with difficulty by this method. In tests made on one animal daily for 7 days the time required for suction to produce hemorrhage varied from 9 to 12 minutes. 50 tests on 20 normal rabbits, designated 1 to 20, produced hemorrhage after the time intervals shown in the tabulation.

5 min.	6 min.	7 min.	8 min.	9 min.	10 min.	11 min.	12 min.	15 min.
Rabbit No.	Rabbit No.	Rabbit No.	Rabbit No.	Rabbit No.	Rabbit No.	Rabbit No.	Rabbit No.	Rabbit No.
11	1	3	2	2	7	8	6	14
	11	3	2	6	9	19	8	
	17	5	3	10	9		8	
	17	14	4	13	19		10	
	17	15	4		19		14	
	20	15	5					
	20	16	5					
	20	18	7					
		18	7					
			12					
			12					
			13					
			14					
			16					
			16					

Histological studies of the lesions produced in normal skin by suction of 8 minutes duration did not determine whether the capillary bleeding in the cutis was due to rupture or to diapedesis of red corpuscles.

Inflammation Produced by a Chemical Irritant

Changes in capillary fragility were studied at the site of inflammation produced by a chemical irritant. Turpentine was diluted with

paraffin oil 1:50, and 0.1 cc. of the mixture was injected intracutane-
ously at 10 sites on the flank of a rabbit. Redness and edema marked
the site of inflammation during a period of several days. Suction
applied to the normal skin produced hemorrhage after 12 minutes,
but 30 minutes after injection suction had to be applied during 100
minutes to produce hemorrhage. Increased capillary fragility made
its appearance 6 hours after injection when the suction time was 2
minutes and 30 seconds, and was still present after 24 hours with a
suction time of 3 minutes. The capillary resistance against suction
was approximately normal 48 hours after injection.
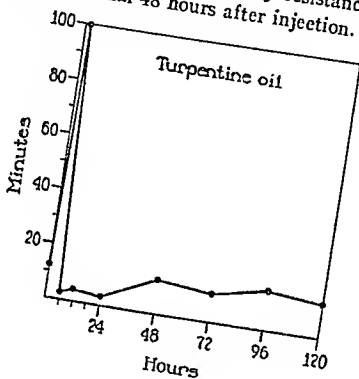
Fic. 2

Inflammation Produced by Bacteria

Inflammation was produced in the first of these experiments by
injection of killed hemolytic streptococci into the skin. To a broth
culture 24 hours old 0.2 per cent formalin was added and after centri-
fuging the sediment was suspended in saline up to the original volume
of the culture. Intracutaneous injections of 0.1 cc. of the suspension
were made at 10 sites. Suction tests were made by applying the suc-
tion tube to the central parts of injected areas at different time
intervals after injection (Fig. 3, 1st injection). The skin became pale
immediately after injection and remained white from 1 to 3 hours.
30 minutes following injection suction produced hyperemia after it

had been applied for 95 minutes and 25 minutes later hemorrhage appeared over the whole area of suction, but it was somewhat less
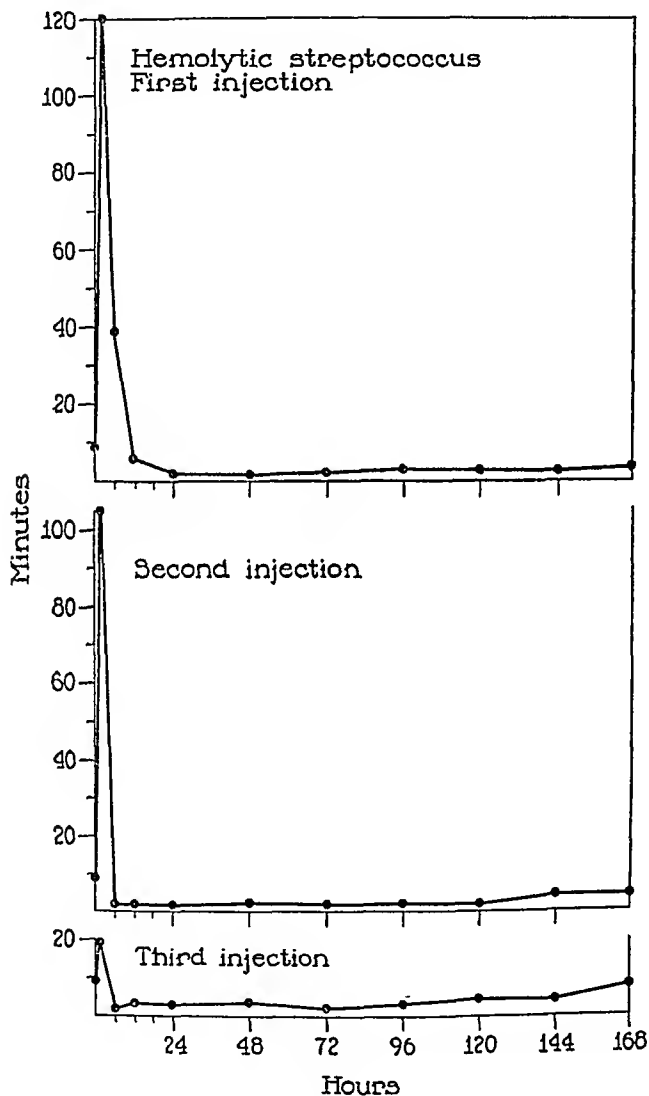
FIG. 3

uniform and usually brighter red than that which occurs in normal skin. There was no difference in the result of suction when a high

vacuum (-300 mm. Hg) or the usual low vacuum (-70 mm. Hg) was used. After 6 hours when redness and edema of inflammation had appeared resistance to suction slightly greater than normal still remained. 12 hours after the injection the suction time was reduced to 6 minutes and after 24 hours to 1 minute and 45 seconds. The increased susceptibility to bleeding with suction persisted for several days. The experiment was repeated on 5 rabbits and showed the same increase in capillary fragility which gradually disappeared after 6 to 9 days.
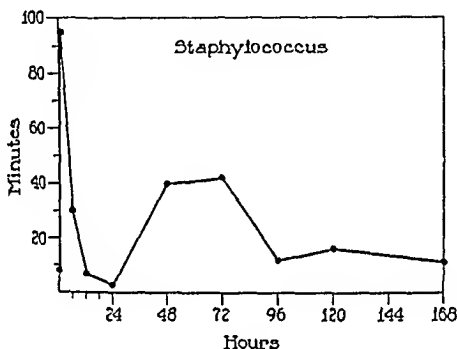
FIG. 4

Inflammation produced by injection of other heat-killed bacteria caused similar changes. A bouillon culture of *Staphylococcus aureus* 24 hours old was killed by heating to 60°C. for 30 minutes and 0.5 cc. was injected intracutaneously at each of 10 sites on the flanks of a rabbit. There was ill defined redness and edema after 24 hours. The suction test applied to the central parts of these lesions demonstrated (Fig. 4) that the time required to produce hemorrhage was greatly increased a half hour after the injection and was diminished below normal after 24 hours. Increased susceptibility to capillary bleeding disappeared after 48 hours. The time required to produce hemorrhage exceeded normal after 72 hours but later returned to approximately the normal level.

Inflammation produced by a suspension of heat-killed meningococci in saline solution containing approximately 2,000,000,000 bacteria per cc. was accompanied by similar changes in the rapidity with which hemorrhage was caused by suction. Intracutaneous injections of 0.2 cc. of the suspension produced redness and edema in 10 areas and these were tested by suction after different intervals following injection. There was prolongation of suction time (75 minutes) preceding hemorrhage 30 minutes after injection and an increase in capillary fragility after 12 and 24 hours with suction times of 2 minutes and 30 seconds and 4 minutes, respectively. The suction time had returned to normal (10 minutes) after 48 hours and after 72 hours
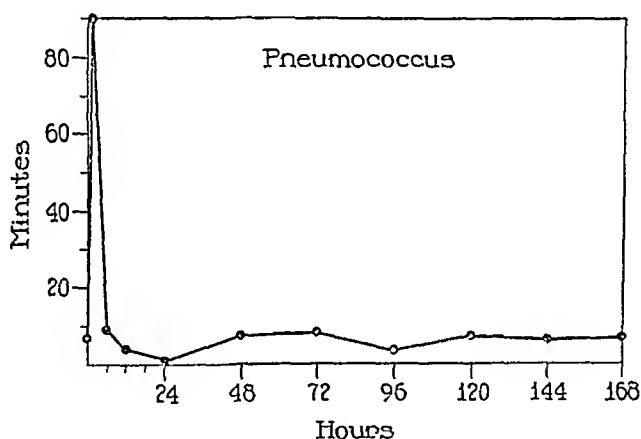
FIG. 5

exceeded this level (18 minutes) but later became approximately normal.

Each of two rabbits received ten intracutaneous injections of 0.2 cc. of a heat-killed bouillon culture of pneumococcus which was originally obtained from a human case and then passed several times through rabbits until it was highly virulent for these animals. The resulting areas of redness and edema after 48 hours measured about 12 mm. across and were elevated 2 mm. The results of the suction tests applied in the center of the lesions at the usual time intervals gave a curve (Fig. 5) similar to those of the foregoing experiments. Capillary resistance returned to normal after 48 hours.

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In all these experiments greatly increased resistance of the capillaries to suction was observed immediately following the injection of a chemical or of heat-killed bacteria into the skin. It is probably due to contraction of the small cutaneous vessels stimulated by the injected irritant. It does not occur when a second injection of an inflammatory irritant is made into the site of acute inflammation, presumably because the vascular walls are paralyzed by the first injection. A heat-killed culture of *Staphylococcus aureus* was injected intracutaneously in a number of places each receiving 0.2 cc., and a test made after 30 minutes demonstrated the usual increase in capillary resistance to the production of hemorrhage by suction. After 24 hours 0.2 cc. of the same heat-killed culture was now injected into an area of inflammation produced by one of the preceding injections. After an interval of 30 minutes the suction test applied to the lesion showed no increased resistance to the production of hemorrhage by suction. The experiment was repeated twice with killed streptococci and the same result was obtained.

Allergic Inflammation

Allergic inflammation produced by repeated injection of an antigen is characterized by rapid appearance and increased intensity of various manifestations of inflammation (Opie, 12). Experiments have been undertaken to determine what changes in capillary fragility occur during the course of allergic inflammation produced by killed hemolytic streptococci.

The six rabbits that were used in the experiment already described (Fig. 3, 1st injection) received 10 days after the first injection 10 intracutaneous injections of 0.1 cc. of a similar streptococcus suspension. The suction test was applied to these lesions after time intervals that were the same as those in the first series (Fig. 3, 2nd injection). Sensitization was indicated by the increased size of the lesions, which after 48 hours were well defined, red and edematous and measured 11 x 10 x 2 mm. 30 minutes after injection there was prolongation of the suction time for hemorrhage only slightly less than that after the first injection. The conspicuous increase in susceptibility to capillary bleeding that appeared in inflamed areas of the first series of injections after 12 to 24 hours was found in inflamed after 6 hours. 10 days later a third

series of 10 intracutaneous injections of the same amount of killed streptococcus was made in each of these rabbits. A half hour after injection, the primary prolongation of suction time required to produce bleeding was much less (Fig. 3, 3rd injection) than that observed in the lesions of the second series of injections, but it is not evident that this inhibition of resistance that ordinarily occurs soon after the injection of an inflammatory irritant is characteristic of sensitization. A more constant change was the rapidity with which increased capillary fragility made its appearance.

Similar changes in capillary fragility were found in another series of experiments in which rabbits were well sensitized to hemolytic streptococci. 9 rabbits received an intracutaneous injection of 0.1 cc. of a suspension of killed hemolytic streptococci daily for a week and no
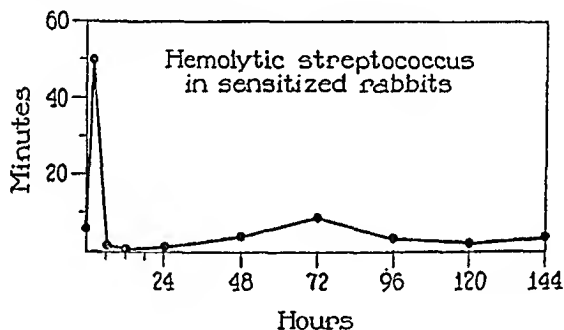
Fig. 6

injections during the following week. A 2nd week of daily injections was followed by a week of rest. During a 3rd week of daily injections suction tests were applied to the lesions that were produced by the first of these daily injections. The lesion of 1 rabbit was tested 30 minutes after the injection, the lesion of another rabbit after 6 hours, of a 3rd after 12 hours, of a 4th after 24 hours, of a 5th rabbit after 2 days, of a 6th after 3 days, of a 7th after 4 days, of an 8th after 5 days and of a 9th after 6 days. The same procedure was applied to the lesions produced by injections on the 2nd, 3rd, 4th, 5th and 6th day of this third series of daily injections. The average extent of redness and edema 48 hours after injection of killed streptococci was 25 mm. in diameter and elevated 5 mm.; necrosis occurred in the central part of some lesions. The test was applied at the edge of the lesions where there was no necrosis.

The graphs representing tests made upon lesions of each of the 6 days were similar. The changes in capillary fragility that occurred in lesions following injection on the 2nd day are shown in Fig. 6. The early appearance of advanced capillary fragility evident after 6 hours was a constant feature of the lesions in these sensitized animals. It was greatest at 12 hours, the whole area subjected to the suction becoming hemorrhagic after 40 seconds. The capillary resistance in these animals returned to normal a few days earlier than in animals that had not been sensitized by preceding injections of streptococci. Injection of tuberculin into animals sensitized by injections of killed tubercle bacilli produced severe and long continued capillary damage.
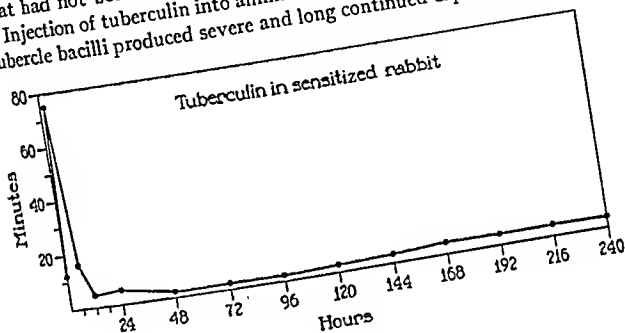

Tuberculin in sensitized rabbit

FIG. 7

2 sensitized rabbits received in the flanks 14 intracutaneous injections of 0.2 cc. of Old Tuberculin diluted 1:5 with physiological saline solution. In one rabbit after 48 hours there was ill defined redness and edema 32 x 22 mm., elevated 1 mm.; in the other rabbit 15 x 12 mm., elevated 1 mm. The results of the suction tests were essentially the same in the two animals. After the usual primary retardation of bleeding, increased capillary fragility made its appearance but was not greater than that with inflammation in unsensitized animals. This increase of capillary fragility persisted during 2 weeks. The last two tests are not shown in Fig. 7.

It is noteworthy that the resistance of capillaries to suction with allergic inflammation undergoes changes that are essentially the same

as those with inflammation in normal animals. In animals sensitized to streptococci increased fragility has made its appearance sooner than in normal animals, but with the reaction to tuberculin in sensitized animals this acceleration has not been found.

Shwartzman Phenomenon

The Shwartzman phenomenon (13–15) occurs when an injection of certain toxic bacterial filtrates into the cutis is followed after 24 hours by the injection of the same substance into the circulating blood. A hemorrhagic lesion occurs at the site of the preparatory injection. Experiments were undertaken to determine if changes in capillary fragility at the site of this skin-preparatory injection render the tissues here susceptible to hemorrhage.
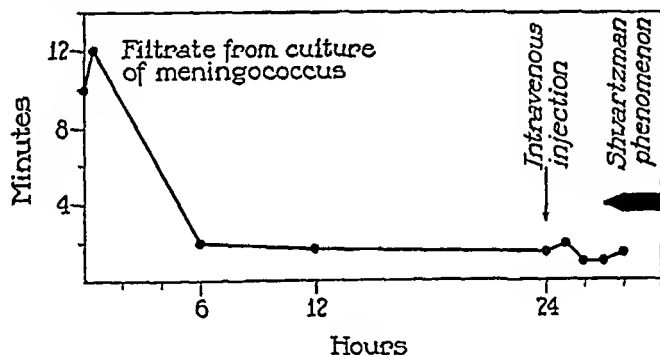

FIG. 8

Meningococcus filtrate was prepared by Dr. Jules Freund according to the method recommended by Shwartzman. 0.1 cc. of diluted filtrate (1 part filtrate and 2 parts physiological saline solution) was injected intracutaneously at 9 sites. Suction tests were applied at the edges of these lesions after it was found that the edema in the center protected the vessels from suction. Prolongation of the suction time 30 minutes after injection was unusually slight. Greatly increased capillary fragility was evident at 6, 12 and 24 hours, the suction times required to produce hemorrhage being respectively 2 minutes, 1 minute and 50 seconds and 1 minute and 20 seconds. At the sites of the preparatory injection after 24 hours edema and redness were approximately 15 mm. across and elevated 1 mm. At this time 1 cc.

of the undiluted filtrate was injected intravenously. 1 hour following the intravenous injection 2 of the 9 injected areas showed a slight purple discoloration and after about 3 hours all of them were the sites of hemorrhage of varying intensity.

In a second experiment in which intracutaneous injections of 0.1 cc. of diluted (1:6) and an intravenous injection of 1 cc. undiluted meningococcus filtrate were used to produce the Shwartzman phenomenon similar increase in capillary fragility was associated with the occurrence of hemorrhagic lesions. In this experiment the preparatory lesions were less edematous and suction applied in the center of them produced hemorrhage ½ hour after injection in 10 minutes, after 6 hours
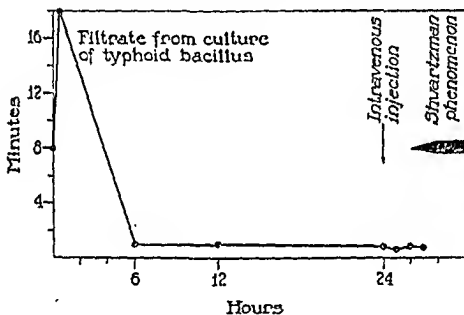

FIG. 9

in 2 minutes and 30 seconds and after 24 hours in 1 minute and 40 seconds.

A similar experiment was made with typhoid filtrate prepared according to the method recommended by Shwartzman. 2 rabbits received intracutaneous injections of 0.2 cc. of the filtrate at 8 sites and the suction test was applied at the usual intervals. The preliminary prolongation of suction time was less than that usually observed. Greatly increased capillary fragility was found after 6 hours, suction time being 1 minute and 30 seconds, although little edema was evident at this time. Unusual susceptibility to capillary hemorrhage could be demonstrated 12 and 24 hours after injection, the suction time after

24 hours being 40 seconds in the edematous lesions now 20 mm. in diameter and elevated 1 mm.   After 2 hours following the intravenous injection of 4 cc. of the same filtrate, purple discoloration began to appear in these lesions and hemorrhage became evident.

The preparatory injection used to produce the Shwartzman reaction produces changes in capillaries that increase their susceptibility to hemorrhage.   These changes follow a sequence that is the same as that observed with other forms of inflammation (Koplik, 15), but capillary fragility produced by substances that elicit the Shwartzman phenomenon is unusually great.   It is probable that the intravenous injection reaches by way of the blood stream injured capillaries at the site of inflammation produced by the preparatory injection and intensifies this injury so that hemorrhage results.

CONCLUSIONS

Suction with a partial vacuum of −70 mm. Hg applied to normal skin of rabbits causes intracutaneous hemorrhage after an average time of 8 minutes.

Inflammation produced by various agents, including turpentine, killed streptococci, staphylococci, pneumococci or meningococci, and filtrates from cultures of meningococci or typhoid bacilli, produces a series of changes that are almost uniform.

Immediately after injection of the irritant there is greatly increased resistance to the production of hemorrhage by suction so that the time required may be from ½ hour to almost 2 hours.   This increased resistance to suction applied to the surface of the skin is doubtless caused by contraction of blood vessels following injection of the irritant.

The period of increased resistance is soon followed by diminished resistance of the vascular walls so that hemorrhage after 12 to 24 hours following injection occurs within from 1 to 4 minutes of suction.

The subsequent course of events varies; resistance of the vascular wall to suction, designated for convenience capillary fragility, may return to normal after from 2 to 9 days (observed with turpentine, streptococcus), or for several days may considerably exceed this level (observed with staphylococcus, pneumococcus), or may remain at a low level for a week or more (observed with tuberculin).

With inflammation in a sensitized animal (allergic inflammation) the preliminary period of resistance may be diminished and the appearance of capillary fragility hastened, so that hemorrhage occurs after 2 minutes of suction applied 6 hours after injection, and later it may fall to an even lower level (observed with hemolytic streptococci in sensitized animals).

The preparatory injection of toxic substances, such as meningococcus or typhoid filtrate, used in the production of the Shwartzman phenomenon, causes inflammation with injury of small blood vessels indicated by susceptibility to hemorrhage with suction. It is probable that subsequent intravenous injection causes hemorrhage by further injury to these injured blood vessels.

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